

Open season for nuclear diplomatists

The world's nuclear diplomatists are in for a long hot summer. Many of them, still recovering from the meeting of the UN Committee on Disarmament which ended only a week ago, will have been back in Geneva on Monday for the Second Review Conference of the Non-Proliferation Treaty, which is planned to last four weeks. Once that is over, many of the same people will be off to Vienna for the annual General Conference of the International Atomic Energy Agency (IAEA), which on past form is likely to be much occupied with the adequacy (and the cost) of the international nuclear safeguards system. This year, however, two weeks in Vienna will not for many be sufficient — they may have to stay on for the first meeting of the new Committee on Assurance of Supply, the body whose existence was decreed in June by the Board of Governors of the IAEA, whose composition and agenda are not yet decided but which could play a crucial part in helping to bolster up the Non-Proliferation Treaty. What, it is natural to ask, will this bout of activity accomplish? Will the families of the diplomatists who will thus be giving up vacations think their sacrifice has been worthwhile?

As always, it is too soon to say, but the prospects for the weeks ahead should be clarified by what happens in Geneva between now and 5 September. Inevitably, the NPT conference will be a quarrelsome affair. Many harsh things will be said, especially by non-nuclear parties to the treaty (see *Nature*, 12 June). Some will complain that the three nuclear powers which have signed the NPT (the Soviet Union, the United Kingdom and the United States) have done too little to implement their promise to negotiate measures of strategic disarmament. If Salt II were not in limbo, the nuclear powers might have been able to reply convincingly. The best they have to offer now is the tripartite report on the negotiations towards a Comprehensive Test-Ban Treaty, submitted to the Committee on Disarmament at the end of July. Plainly, there has been some progress, but a cheerful interim statement is no substitute for a treaty. That is what the non-nuclear parties to the NPT will be saying. The same states will complain that too little has been done, both by the nuclear powers and those signatories of the NPT in a position technologically to do so, to foster the development of civil nuclear power elsewhere. The nuclear powers are no doubt hoping to blunt the edge of this complaint by referring to the meeting planned for Vienna at the end of September. They must expect to have to make some promises about the scope and speed of those proceedings. But the test by which the success of the Review Conference should be judged is that of whether the non-nuclear signatories of the NPT leave Geneva having been persuaded that non-proliferation has come to stay — or at least that the present regime will last for another five years.

It is therefore important that, in at least two respects this second conference will not be much occupied with two of the issues that perplexed the first, in 1975. Then, the non-nuclear signatories of the treaty were still grumbling about its discriminatory character. Is it not an outrage, they were inclined to ask, that safeguards should be rigorously applied to non-nuclear states but not to the real black sheep, the nuclear powers themselves? The treaty has not changed in the past five years, but it does appear that all parties to the treaty have come to accept that it must be discriminatory in this narrow sense. It cannot be otherwise unless and until the nuclear powers have agreed to forsake nuclear weapons altogether. With the fate of Salt II in mind, few at Geneva will be asking for that moon now. The vigour with which they would have complained at discrimination will no doubt be

diverted into the more legitimate complaint that the nuclear powers, having dragooned and cajoled non-nuclear powers into signature of the treaty, have then chosen to impose separately agreed restrictions on international trade in nuclear equipment even when the would-be recipients have signed the NPT and come to safeguards agreements with the IAEA.

The other bone of contention which has been resolved in the past five years is the issue of peaceful nuclear explosions. Largely at the insistence in the 1960s of countries such as Brazil and India (which, ironically, is not a signatory of the treaty), the NPT requires that the nuclear powers should provide, through the IAEA, peaceful nuclear explosions to those non-nuclear powers able to persuade the IAEA that they have a need of them. Mercifully, the suspicion that peaceful nuclear explosions would be an economic blessing in disguise is fast melting away. Only in the Soviet Union, where as many as eight of the 28 nuclear explosions in 1979 may have been "peaceful", does enthusiasm persist. The government of India, which used the PNE argument to justify its nuclear explosion in May 1974, has not thought it worthwhile repeating its indiscretion. The nuclear powers need not expect a drubbing at Geneva because they have not been trudging the world for the past decade, offering to blow out harbours and other holes in the ground for all and sundry.

For small mercies, the nuclear powers will no doubt be grateful. What can they say on the major issues they will be confronted with? By now, fortunately, there is enough experience of the NPT for its true nature to be widely understood. The treaty itself is no more a guarantee that anxiety about nuclear proliferation can be banished than was Prohibition a guarantee against alcoholism. From the start, the principle underlying the treaty has been that of a bargain between the nuclear and non-nuclear powers. The nuclear powers would work towards strategic arms control, the non-nuclear powers would meanwhile not embark on military programmes. The bargain is, of course, not comprehensive. China, France and India remain outside the treaty, as do a great many supposedly non-nuclear powers that are presumed to have an interest in nuclear weapons — Pakistan, Brazil, Argentina, Egypt, Israel and South Africa, for example. Worse still, some of the signatories of the NPT are rightly regarded with suspicion. Is it possible that countries such as Libya or Iraq, dutiful signatories of the NPT, have it in mind that they can use the benefits of access to nuclear technology which the treaty offers so as to build up a stock of fissile material and then simply give notice, as the NPT allows, that they are withdrawing from its provisions and its safeguards? In such circumstances, it is wisest that even the staunchest supporters of the NPT should recognize its weaknesses. The only realistic objective is somehow to contain the pace of nuclear proliferation — which, paradoxically, requires that the NPT system should be robust enough to survive the emergence of the next nuclear power, whichever it may be. At the same time, it is essential that states not yet party to the treaty should be provided not merely with a sense of pressure from the non-proliferators but also that they should be given incentives in due course to sign on the dotted line.

This is the sense in which the meeting of the Committee on the Assurance of Supply at the end of September may be crucial. Unlike the review conference of the NPT, the committee will in principle bring together all members of the IAEA, France and China among them — although nobody knows as yet which will turn up. This broadening of the scope of discussions about proliferation is greatly to be welcomed. The committee's most



urgent task is to restore the confidence of the non-nuclear states in the willingness of potential suppliers to sell nuclear equipment and raw materials. President Carter's unilateral out-of-the-blue non-proliferation policy of 1967, and the tactless US Nuclear Non-Proliferation Act of 1968, has been the chief but not the only offender. The US Administration's difficulty in living with the Act it has sponsored in Congress (see *Nature*, 31 July) should be a sign to people elsewhere that there is no future in an agreement among suppliers to trade only with states that have accepted full IAEA safeguards. The government of France would not even consider such a proposal. The best hope for the CAS meeting is to devise incentives that will persuade states outside the NPT system that it would be in their best interests to join the club. An international convention to regulate the nuclear business on a multilateral basis, however desirable, is probably for similar reasons unattainable. Uranium banks under international auspices, or internationally managed reprocessing plants, offer more scope for imaginative negotiation. And is it too much to ask that the nuclear powers and the nuclear suppliers can use the two months ahead to repeat the message they were trumpeting a quarter of a century ago — that nuclear power is indeed a way of helping forward economic development in all kinds of states? One of the other circumstances that have changed in the past five years

is that non-nuclear states are now in a mood again to believe the message.

The question remains of what the nuclear powers will have to report at the review conference about progress towards strategic disarmament. Last month's report to the Committee on Disarmament shows that the Comprehensive Test-Ban negotiations have made substantial headway toward verification. If the treaty is signed, there will be arrangements for the exchange of seismic data, a committee of "experts" to assess them and procedures for dealing with challenges raised by one party or another. So much has been agreed. So why not sign the treaty right away? That is what some non-nuclear powers will be asking in Geneva in the next few weeks. In doing so, they will enormously underestimate the problems with which the three nuclear powers are grappling. States such as the Soviet Union and the United States are considering, apparently in good faith, binding themselves to forswear the further development of nuclear weapons, thus ensuring that, in the course of time, weapons now considered central to their security will be built less confidently than at present — and are doing this while there remains no assurance that China and France would follow suit. It is a huge political enterprise — and one that may not succeed. To have travelled so far along such an unpromising road is creditable.

Putting Finniston's cart before the horse

The British government's response to the Finniston report on engineering education is best described as frivolous. That may have been inevitable. The report was commissioned by a Labour government with an earnest conviction that governments have a duty to ensure that social institutions of all kinds are well suited to their purpose. Sir Monty Finniston and his colleagues on the committee of inquiry, set up in 1978, responded with gusto to the implicit invitation to reconstruct the whole pattern of engineering education. They were nothing if not radical. They set out to diminish the influence of the engineering institutions by proposing that there should be a new body, called the British Engineering Authority, to be responsible both for the certification of professional engineers and the validation of engineering courses, in universities and elsewhere in higher education. The committee also asked for changes in the educational system itself; engineering students would follow courses much like those now available, but with more work experience, but a minority would stay on for an extra year and come away with a higher qualification. The underlying objective of the Finniston Committee was to enhance the reputation of engineers in British society as a whole, whence (in part) the proposals that the state should pay larger stipends to engineering students than to others, that public authorities should not let contracts without insisting that only registered engineers are employed on them, and that properly registered engineers should be entitled by statute to regular periods of sabbatical leave (admittedly in the good cause of continuing education). Looking back over the past two years, and at the host of these fondly conceived but now stillborn proposals, the committee will no doubt rue its bad luck. It has fallen to a non-interventionist government, and to the most zealously non-interventionist minister of that government (Sir Keith Joseph) to respond.

An engineering authority of some kind will indeed come about, but not yet and not in the form the Finniston Committee asked for. Certainly it will not be the body "with teeth" that Sir Monty Finniston and his colleagues have been asking for since their report was published. Instead, the Privy Council (which can grant Royal Charters and thus legitimize the activities of non-governmental public bodies, universities for example) will be asked to set it up. The government will appoint the first slate of members, and help with petty cash for the first year or two. After that the authority will be expected to make its own way in the world, persuading universities that it is in their interests to have

their courses validated by the new organization, qualified engineers that they had better be registered under the new rules (still to be written) than with the existing engineering institutions and employers that the new certificate of registration is a worthwhile professional qualification. The prospect is daunting. There is a high chance that the new authority will be no more successful than the Council of Engineering Institutions, set up for similar purposes by the engineering institutions themselves (and which, apparently, will continue to exist, no doubt sniping from the wings from time to time). Many will say that it would have been better not to have had an engineering authority at all than to have so willingly courted the chance of failure. And that is the sense in which the government's decision is frivolous. For whatever the faults of the Finniston proposals, and there are several, the new arrangements are certain to be regarded — as was the Finniston Committee itself — as the best hope for the improvement of the engineering profession. Once the new authority exists, it will for a time at least tend to stifle other instruments of change.

This is a sad solution to have been wrought from the widespread recognition, in the past few years, that something should be done to change the pattern of engineering education and professional recognition. The explanation seems to be that first Finniston and now the government have put the cart before the horse. The most urgent need is for a more imaginative experiment in engineering education in the universities, and for level-headed evaluation of the consequences. Everybody accepts that intending engineers, like intending doctors, profit from first-hand knowledge of the problems with which they will have to deal in later life. There is, however, no assurance that the pattern of four-year courses now being tried out in English universities (and which Finniston endorsed) will be a substantial improvement on the present. To the extent that these courses will continue to skimp on basic science, the result may well be engineers whose education is more expensive but who are no better fitted for the needs of modern industry. For the truth is that a profession such as engineering should be a cadre of people with very different backgrounds and skills. The objective would have been better served if the government had encouraged the universities, through the existing machinery of the University Grants Committee, to develop courses that differ among each other but which collectively might yield the diversity the profession needs. The trouble is, of course, that such a development would require extra funds for the universities. This is hardly the time to be asking for more.

Another violation of NIH guidelines

Washington

Cross-contamination between vials containing viruses being transported from Britain to California may have caused a San Diego scientist inadvertently to carry out experiments prohibited under the National Institutes of Health's guidelines for recombinant DNA research. This, at least, is the "working hypothesis" which Dr Samuel I. Kennedy, of the University of California at San Diego, offered last week to explain what may prove to be the third violation of the guidelines.

Dr Kennedy, who moved to California from the University of Warwick in 1977, has been studying the activity of several arboviruses. In particular, he has been looking at the ways in which virus reproduction is inhibited by naturally produced "defective interfering particles" containing only a fragment of the virus genetic material. Earlier this year, Dr Kennedy and his research assistants cloned what they thought were subgenomic fragments of the Sindbis virus, a cause of fever and skin rash carried by mosquitoes in parts of Africa, India and Australia.

Following "confusing" results of attempts to characterize the nucleotide sequences in the resultant recombinant DNA produced in disabled *Escherichia coli*, the cloned material was sent to the Public Health Laboratories in Berkeley for analysis. There it was discovered that the cloned fragments appeared to have derived not from Sindbis virus but from Semliki Forest virus, another arbovirus being studied in Kennedy's laboratory.

This raised difficulties, for although the Sindbis virus has been classified by the Center for Disease Control (CDC) as a Class 2 agent — which under the NIH guidelines could be cloned under P2 physical containment conditions — the classification of the Semliki virus was ambiguous.

Until recently, the Semliki virus, known primarily as a cause of headaches and fever, was considered even safer to work with than Sindbis virus. Three years ago, however, a German research worker died after being infected by a wild-type strain of the virus. As a result of this incident, CDC is considering classifying the Semliki Forest virus as C3, although subject to certain qualifications.

At the end of last month, NIH announced changes in their guidelines to allow viruses in this class to be cloned under P3 containment conditions. However, at the time when Dr Kennedy's experiments were carried out, the cloning of C3 agents was prohibited.

As soon as the university suspected that the NIH guidelines might have been violated, both the Institutional Biosafety Committee and the NIH's Office of Recombinant DNA Activities (ORDA) were informed. ORDA is now carrying out

an investigation, and will eventually make recommendations to NIH Director Dr Donald Fredrickson on what action should be taken.

The ORDA investigation seems likely to concentrate on three aspects. First, how could the confusion between the two types of virus have occurred and was this the result of cross-contamination during transit from research laboratories in Britain?

Second, ORDA must determine what classification it would have given to the Semliki Forest virus if a cloning application had been made at the beginning of the year. Approval as a Class 2 agent would have permitted the experiment; approval as Class 3 would have disallowed it. This problem is complicated by the fact that CDC classification of arboviruses is under revision. A European scientist was told by NIH earlier this year that although the Semliki virus would probably be considered to fall within Class 3, in certain geographic locations and conditions of use it might be considered C2, permitting cloning under P2 containment.

Finally, ORDA will be looking closely at the procedures followed by Dr Kennedy and the university once a violation of the guidelines was suspected. Dr Kennedy

stopped work on the cloned fragments, and informed the Institutional Biohazard Committee of the apparent mix-up, as soon as the results of the laboratory tests were received at the end of last month. Some of his graduate students, however, had previously approached the head of the biology department, Dr Donald Helinski, expressing concern about preliminary results and taking a different view of the actions that ought to be taken.

Both university and NIH officials stress that there was no public hazard involved. "We were not even cloning the entire genetic information in the virus" says Kennedy, pointing out that even if it was Semliki virus, the guidelines allow this to be safely cloned under the P3 containment conditions that were in fact used.

The biggest impact of the incident is likely to be on public perception of how the guidelines are working. Since the NIH guidelines were introduced in 1976, there have been two reported violations. In one a scientist from the Harvard Medical School carried out experiments without the necessary prior clearance from NIH. In the other, San Francisco scientists conducted experiments with a plasmid vector before it had been officially approved.

David Dickson

Small beer for scientists

Scientists working for the British Civil Service are unlikely to be satisfied by the outcome of the arbitration on salary scales for 1980, which was completed last week. After the arbitration award was made known, Mr William McCall, General Secretary of the Institution of Professional Civil Servants (IPCS), said that the outcome was disgraceful and that "if this is what the system now in force for settling the pay of scientists in the Civil Service produces, then the system will have to be changed".

There have been several bones of contention between the IPCS and the Civil Service Department since the first offer of salaries for 1980. The department's proposals (see *Nature*, 24 July) were especially mean to the higher grades of the scientific Civil Service — those in the grade of Senior Principal Scientific Officer were, for example, offered no increase except the annual increments to which they were contractually entitled.

The IPCS has complained consistently that the device of pay research for relating the salaries of higher civil servants to those of comparable groups of workers in the private sector undermines the principle that scientists within the Civil Service should be paid the same as other civil servants of comparable rank and that, in any case, the data on salaries earned by scientists in the private sector are insufficient as an accurate foundation for

pay research.

The Civil Service Arbitration Tribunal appears to have gone only a little way to meet these protests. The salaries of Senior Principal Scientific Officers will after all be increased by 3.2 per cent in 1980 compared with 1979, taking the maximum salary in this grade to £16,250 a year, compared with the claim by the union for £18,248.

The salaries of Principal Scientific Officers, which ranged from £8,613 to £11,343 at the beginning of the year, are to be increased to between £9,690 and £12,540, figures which are increased by between 1.5 per cent and 4.1 per cent as a result of the tribunal's intervention.

Scientific Officers, earlier in the year earning between £6,332 and £8,705, are now to receive salaries increased by between 20 per cent (at the bottom of the scale) and 10 per cent (at the top). The salaries of lower grades will be increased by proportions not very different from the rate of inflation.

Even so, this will not go far to meet the complaint that the salaries of scientists in the Civil Service are less than those of people of similar rank — Principal Scientific Officers will, for example, be paid £1,260 a year less than Administrative Officers.

The reason why the arbitration tribunal made this new award are not known, and may never be, although a report on the issue will be published next month.

Nuclear Iraq

Perrin protests

Anxieties about French nuclear collaboration with Iraq have now burst into the open. Last week, the grand old man of nuclear power in France, Francis Perrin, contradicted his government's statements about the supply of a research reactor and highly enriched uranium to Iraq, in a deal to exchange oil for technology. "It cannot be said", Perrin argued in an interview with *France-Soir*, "that the provision of this reactor will not enable a country like Iraq to produce an atomic weapon in a few years' time".

Without addressing the question directly, however, the French government had earlier insisted that its cooperation with Iraq is directed to "perfectly legitimate ends", and that the agreement is also covered by guarantees. The government said it was "astonished" at the accusations that had been made against it, and asked by what principle Iraq's right to nuclear power could be refused.

Perrin, who was president of the Commissariat à l'Energie Atomique from 1951, and who was confirmed in that post by President de Gaulle in 1960, does not believe that nuclear peace and war can be separated so neatly. He believes it unlikely that Iraq would directly divert any part of the 11-kg charge of 93 per cent uranium-235 to be supplied by France, for this would fly directly in the face of too many international agreements. "But evidently, if Iraq trains personnel with this reactor, they could prepare plutonium by irradiating [unenriched] uranium." This plutonium might be prepared initially for research; then, when they had enough plutonium and technicians, they could break their agreement (under the Non-Proliferation Treaty, which Iraq signed in 1969) not to build weapons.

The controls of the International Atomic Energy Agency — which requires monthly flow-sheets of fissile materials belonging to NPT signatories, and whose inspectors make regular visits to their nuclear establishments — are effective, says Perrin, but a country can always decide to reject them.

Iraq already has a small Soviet reactor, using 80 per cent enriched fuel, under IAEA safeguards. The details of the new French reactor have not yet been received at the IAEA, but the safeguards provisions require that they should arrive more than six months before the reactor is charged with fuel. Charging is, however, unlikely before the end of 1981, the date set before Iraq's chief nuclear expert, Yihya al Meshad, was murdered in Paris on 13 June. It is, however, known that the reactor will be based on the French 50-MW thermal reactor Osiris at Orsay, which takes a charge of 9½ kg of 93 per cent uranium-235. Osirak, the Iraqi version, is planned to be slightly bigger — 70 MW with an 11-kg charge.

Italy is also supplying nuclear facilities to Iraq in exchange for oil, including an isotope laboratory with a hot cell which in principle could be used for reprocessing and plutonium separation but, says the Italian government, it is much too small to have a bearing on weapons production.

President Saddam Hussein of Iraq has rejected all speculation that Iraq may be building a weapon, and has pointed out that Israel is generally regarded as already having the bomb, and has never signed the NPT. "Other countries" said an IAEA spokesman "have been less cooperative than Iraq".

President Hussein, with a \$75,000



Troubled seer

million five-year plan, also dreams of a technological transformation of his country, and recognizes that Iraq's principal need is technically trained manpower. He believes that Osirak will contribute to such a pool. Now that Iraq is increasingly turning from its East European and Soviet partners towards OECD countries for technology, it may be wise to take Hussein seriously.

Robert Walgate

Remote sensing

Going private?

Washington

Fierce arguments have been generated in Washington by plans under discussion in the Carter Administration to create a fully operational system from the remote-sensing satellites developed by the National Aeronautics and Space Administration.

Few challenge the idea that responsibility for remote sensing should eventually pass to the private sector, but there is disagreement about the pace at which the transfer should occur and the degree of control which the federal government should retain.

Responsibility for coordinating the transfer from NASA to the private sector has been given to the National Oceanic and Atmospheric Administration (NAOO). The NAOO proposes a two-stage

process, involving an initial stage for developing the sensor technology, that would extend to the end of the decade, before a fully operational system would be available for private use.

Several companies, keen to establish themselves in a potentially lucrative market, argue that this time scale is unnecessarily slow. In particular, executives from the Comsat Corporation, which has already successfully taken over responsibility for telecommunications satellites, are arguing that it could start doing the same for remote sensing by 1983.

The temptation to use telecommunications as a model for successful commercial exploitation is strong, but remote sensing is likely to have a rougher ride. The market is undeveloped and heterogeneous.

Thus at a Senate hearing two weeks ago, a spokesman for the National Governors Association said that the remote-sensing system "should be considered in the same context as census, cartographic, geological and meteorological data which are provided as a public service of the federal government".

Aware of this public responsibility, NOAA is considering two ways of allowing federal agencies to buy data under guaranteed purchase contracts. One would transfer responsibility for this entirely to a private corporation, the other would set up a new "for-profit" corporation with both privately and publicly appointed members.

The cost of remote sensing is another problem. According to NOAA administrator Dr Richard Rank, the remote sensing system will probably not be self-financing until the end of the century, and thus will rely on subsidy for the foreseeable future.

But there will still be a temptation to maximize returns, even in the early stages, and state governments are worried that high prices could discourage potential state and local government users from even exploring the potential usefulness of Landsat data, particularly if — as NOAA has suggested — experimental data during the interim period are priced the same as operational data.

Excessive pricing could also have important international implications. Some countries could complain that they are unable to afford information about their own natural resources which can be bought on the open market by multinational mining corporations. The State Department accepts the principle that prices should be consistent for both domestic and foreign users, but warns that price increases should be gradual so that foreign users can adjust to them.

Present users are, however, more concerned at the quality of the service. NASA now has two Landsat satellites in orbit, but both are having problems which have restricted the amount of data being received. Unexpected difficulties have also arisen in the next satellite, Landsat D,

scheduled to be launched in 1982. Development of the instrument called a "thematic mapper", and intended to provide direct information on crop differentiation, is so much delayed that Landsat D may have to be launched without it.

The delays in both technical plans and administrative arrangements have created inevitable frustrations. Those who have already experienced the benefits of remote sensing technology are keen to exploit it as rapidly as possible, and are already hinting that they may look to other countries — such as France and Japan — as an alternative source of remote sensing services. Moreover, some members of Congress claim that the delays reflect a general lack of imagination in the White House about the US space effort.

NOAA is at present working out the details of the transition plan in preparation for its submission as part of the 1982 budget request early next year. It seems more likely to include only limited private involvement — perhaps restricted to processing and marketing the data produced by federally operated satellites.

David Dickson

Lords committee

Dumping ahead

The House of Lords Select Committee on Science and Technology embarked on its summer break last week with the promise of a new inquiry in October into the disposal of hazardous waste. The inquiry will be the committee's second quick investigation — the report of the first, on electric vehicles, is due to be published at the beginning of September. A longer investigation of forestry in the United Kingdom will continue until the end of the year.

The suggestion of an inquiry into hazardous waste disposal came from Basildon District Council in Essex, where a large proportion of Britain's toxic industrial waste is dumped. When the House of Commons Select Committee on the Environment declined to take the matter up because of other commitments, the Lords committee stepped in, believing the topic to fit well into its remit of investigating areas of science and technology of public concern.

The worry of Basildon District and Essex County Councils is that there is no coherent national policy for hazardous waste disposal, largely because the relevant section of Part II of the 1974 Control of Pollution Act has never been implemented. The councils also believe that Essex has become a main centre for hazardous waste disposal by historical accident rather than for good practical reasons.

The government has laid down guidelines for dealing with the disposal of hazardous waste but no regulations as to who should be responsible for arranging disposal. The 1974 Act says that local authorities should take that responsibility and should inform



Dumping at Pitsea

the Secretary of State for the Environment of the arrangements they have made. The Act empowers the Department of the Environment to bring these and other provisions (affecting water quality, for example) into force when it chooses. So far it has chosen not to do so, allegedly on the grounds of cost. Redland Purle, Britain's largest waste disposal company, owns the country's largest dump at Pitsea in Essex. The council has power to monitor the sites and has taken measures to restrict the quantities dumped, but it would take other local authorities to take some of the load.

The Lords committee will no doubt be looking at the institutional arrangements for coping with hazardous waste. It also intends to investigate the basis for some of the technical arrangements, for example the criteria used for choosing suitable sites and deciding what can be dumped where. The Select Committee hopes the inquiry can come to grips with new methods of disposal and suggest how more use could be quickly made of them.

Judy Redfearn

Energy alternatives

German plans

A West German commission on "future nuclear energy politics", set up two years ago by the Bundestag to resolve the nuclear question, has reached its eagerly awaited conclusions. They are equivocal.

West Germany should shelve a final "decision" on nuclear power until 1990, says the commission. But in the meantime the country should build as many reactors as it needs, provided it makes due effort on the development of coal, conservation and renewable sources.

The all-party parliamentary commission, consisting of seven members of the Bundestag and eight technical experts from industry, environmental groups and the trades unions, considers that it will be impossible to judge — for ten years — whether nuclear energy should be expanded in Germany. The growth rate of electricity consumption, the effectiveness of conservation measures and the impact of renewable energy are too uncertain to make commitments to any particular source.

The commission does however spell out its demands for immediate and increased government powers to enforce energy conservation — the abolition of mileage rates for business cars, an energy tax, more bicycle paths and tighter controls on insulation standards in new homes and factories. The commission also asks for an "energy service" to provide, free of charge, an infrared survey of buildings to show where the heat leaks out. These and other measures have been described by the Christian Democratic Union, the main opposition party, as introducing "a totalitarian energy conservation state".

Elsewhere, the recommendations have been seen as vague and offering all things to all men — especially with the recommendation that reactors should be built if energy demand requires them. The major nuclear construction company in Germany, Kraftwerk-Union, has argued that it will be running out of work in 1983 — it has lost its contract for four reactors in Iran, and there is doubt as to whether Brazil will take up its full option for eight. Thus some suggest that a construction programme of one or two reactors a year over the next decade, a programme similar in its scale to the British, is quite consistent with the commission's view.

At the same time, the report has been interpreted by environmentalists as recommending a further delay in the country's nuclear development. (Germany now has five power reactors above 500 MW electric; in total 15 reactors produce about 9 GW.) It is not surprising that the commission has been represented in the German press as a man wearing two badges, one saying "nuclear no thanks" and the other "nuclear power — yes please".

The forthcoming October general

election may have played some part in the commission's equivocation: there has been an understanding among the parties that nuclear power should not be an election issue. The debate on the report was eclipsed the same day by another on Chancellor Schmidt's visit to Moscow.

The commission also recommended that it should reconstitute itself after the election, to complete its task. It was set up in December 1978 after a lengthy debate on the future of the Kalkar 300 MW prototype fast breeder, now under construction. It was decided then to construct Kalkar, but not to charge it with plutonium until expert advice had been received on Germany's need for a "plutonium economy". The nuclear energy futures commission was set up to provide that advice, but (beginning work in 1979) it decided that there was not time before the election for such a broad investigation. Hence the fast breeder is not mentioned in the commission's recommendations.

The result is that Germany remains without a strong federal lead on nuclear power. It will remain so until the majority of the ruling coalition — only ten in the present Bundestag — cannot be threatened by the strong minority in the coalition which will always vote against nuclear development.

The election is thus critical for nuclear energy, despite the parties' intention to keep it out of the limelight. A number of decisions await a nuclear majority, not the least of them the location of intermediate storage facilities for spent fuel. The CDU Prime Minister of West Saxony, Herr Albrecht, where the Gorleben salt-dome offers a potential long-term geological store, is hoping to give hospitality to the waste after the election. Other current battles (against environmentalist opposition) such as that to increase the density of spent fuel storage, by a factor of four, on reactor sites, by inserting cadmium sheets between the rods to absorb neutrons, may be more easily won if a federal government is returned which can give a more definite "yes" to nuclear power.

Robert Walgate

Pesticide safety

No numbers

Washington

Stepping out of line with the fashionable wisdom — at least among congressional legislators — a little-noticed report from the National Academy of Sciences has come out with some harsh words about the dangers of squeezing data on the health effects of agricultural pesticides into a neat cost-benefit mould.

The report was prepared by a committee of the academy's Environmental Studies

Board as a follow-up to earlier studies by the Environmental Protection Agency (EPA). In general it supports the agency's concern that environmental regulations should be as explicit, and as well quantified, possible.

It also points out, however, that in attempting to produce legally defensible decisions, agency administrators often underplay the uncertainties and qualifications that surround scientific assessments of risks and benefits, particularly of human morbidity and mortality data. Here, the report says, quantification is carried to "unwarranted extremes".

According to the committee, present understanding of cancer development "does not permit us to draw reliable numerical inferences from the kind of laboratory data normally available about the effects of pesticides and other compounds on the development of cancers in humans".

There is therefore a danger, it says, not only of overestimating risk by the use of too conservative assumptions, but also of underestimating it by neglecting potentially important factors. "We say the error can go either way" said committee chairman Professor Robert Dorfmann of Harvard University's department of economics.

The committee therefore recommends that the EPA abandons its attempts to produce numerical estimates of the impact of pesticide use on human health except where reliable epidemiological data are available. Instead, it proposes the use of "a carcinogenic activity indicator" as a measure of relative carcinogenicity of chemical compounds. This would be based on the results of laboratory bioassays.

In addition, to underscore the uncertainties in risk evaluation, two figures are proposed for every risk estimate. One would be a measure of "most probable" risk, conveying the analyst's best judgement of the level that would be realized in assumed conditions. The other would be a "maximum plausible" estimate taken as the upper limit of a 90 per cent confidence interval.

The EPA has made no official comment on the report. The agency is involved in reviewing its procedures for weighing the risks and benefits of pesticides in the light of recent administrative changes.

Its review could be influenced by an unintended consequence of the new report. As part of its analysis, the committee carried out a detailed review of chlorobenzilate, using its recommended procedures to produce its own assessment of the risks and benefits. Comparing its evaluation with that obtained by the EPA in 1976, it concluded that not only had worst-case estimates of exposures used to assess health risks been highly improbable, but also that the economic benefits of using the pesticide — particularly the costs of not using it — had been wildly overestimated.

When a draft version of the report was circulated last year, these discrepancies were seized on by the Environmental Defense Fund (EDF) to challenge the EPA's decision to allow limited use of the pesticide. However, an administrative court in Washington has since ruled that the EDF had no legal standing to take part in EPA's rule-making procedure. Nevertheless, this could have significant impact on rule-making in the agency, since in the past many of the restrictions on pesticide use have followed challenges from the EDF and other similar environmentalist groups.

David Dickson

Russian plate tectonics

Drift of change

"Plate tectonics" and "continental drift" have been dirty words to orthodox Soviet geophysics for many years — orthodoxy being, in this case, laid down not by Party doctrine but by the personal views of Academician Belousov, the acknowledged head of the Soviet geophysical establishment. Rather than let themselves be shut off from the international scientific community, as were the Soviet biologists in Lysenko's day, Soviet geophysicists have become adept in inventing semantic variants such as "ocean-floor movement" under which they can discuss the proscribed ideas.

These strategies have been used, for the most part, strictly within the academic community, and it was only on extremely rare occasions that any mention was made of such concepts in the general press. (One such was the announcement that owing to crustal movements the BAM Baikal-Amur Mainline railway, would be when completed, 50 cm longer than originally planned.)

Now, however, an official of the Soviet Academy of Science's Institute of Archaeology has given what amounts to a press conference on continental drift — though without mentioning any controversial terms. Describing a new map of the lithosphere under the world ocean's prepared by the Institute, the official explained to a TASS correspondent that "the lithosphere is thinnest in the centre of the oceans where median oceanic ridges pass" and that "it is precisely in those areas that the lithosphere is formed."

Information on the thickness and structure of the lithosphere under the oceans is therefore "important for solving many theoretical problems", in particular, for understanding the process of formation of our planet.

Vera Rich

Vietnam in space

Queue-jumping

The recent 8-day sojourn of Vietnamese cosmonaut Pham Tuam aboard the Salyut-6 space station marked a significant change in the Comecon Interkosmos joint space programme. For the first time the non-Soviet participant came from a developing and non-aligned country — a point which both the Soviet and Vietnamese press agencies stressed at the expense of comment on the scientific content.

Pham Tuam's flight, moreover, meant a change in the projected running order of participation, which, most Baikonur watchers had accepted, was related to the size of the contribution the various countries had made to the earlier unmanned stage of the Interkosmos programme (the Interkosmos satellites and Vertikal high-altitude probes). In effect, this meant that the European nations of the bloc would go first, with Cuba and Mongolia bringing up the rear. Vietnam, which joined Comecon in summer 1978, long after all the other members had their cosmonaut candidates installed at the Gagarin space centre, seemed destined for the final place in the programme.

However, with four countries still to go (Romania, and the three non-European members), Vietnam was unexpectedly advanced three places. Both TASS and VNA stressed unequivocally that there were political implications in this move. Whatever the reason for the changed schedule, however, the interesting question remains: what, precisely could Vietnam contribute to the space programme?

It would be incorrect to suggest that the developing members of Comecon — Cuba, Mongolia and Vietnam — have nothing to offer the Interkosmos programme, or at the most can offer only sites for tracking stations. As early as 1978, the Polish cosmonaut Mirosław Hermaszewski said that Cuba and Mongolia were working on their experimental programmes for when their turns came. (At that time, Vietnam had still not joined the Interkosmos programme.) Pham Tuam's programme, as reported to date, corresponds remarkably well with those of the other non-Soviet cosmonauts.

First, and inevitably, came the medical programme, the testing of cardiovascular and lung functions under zero gravity. Then, equally routine, experiments on semiconductor crystal growth — in this case, gallium phosphide and a triple bismuth-antimony-tellurium compound. These experiments use a Soviet furnace aboard Salyut-6, but the test capsules come from the non-Soviet participant, which also provides a suitable symbolic name for the project — Morava for Czechoslovakia, Berolina for East Germany and so on. This time, it was Halong, after a gulf in Vietnam.

Not surprisingly, the regular photographic and visual observations carried out from Salyut were given a Vietnamese slant. Silting up of the river mouths, and the effect this has on fish feeding habits, observations of tidal flooding and erosion, and hydrological surveys of the Mekong and Red River Basins were especially stressed. And, most Vietnamese of all, Pham Tuam had taken along samples of azola water ferns, fast-growing, nitrogen-rich plants which are traditionally used as fertilizers for the growing rice crop, and which could prove useful aboard future permanent space stations.

Comecon doctrine concerning its third world projects (including its own three "developing" members) firmly eschew the concept of "aid". All such undertakings are termed "cooperation" projects, although recent bilateral scientific-technological cooperation deals signed between Vietnam and, for example, Czechoslovakia and East Germany consist largely of the European partner providing experts and know-how, and Vietnam reciprocating with raw materials. Nevertheless, no attempt has been made, as far as Interkosmos is concerned, to present the joint Soviet-Vietnamese flight as a partnership of technological equals. While by no means a passenger, Pham Tuam characterized his role rather as a harbinger of things to come. "It is a new demon-

stration of the effective cooperation within the socialist community", he said before embarking, which "will also demonstrate the abilities of the Vietnamese people".

Vera Rich

Airport noise

London's out

The Noise Advisory Council (NAC) has provided some timely ammunition for the opponents of the plan to build the third London Airport at Stansted in Essex. Last week, hard on the heels of the formal application for permission to develop the airport by the British Airport Authority, the council announced that the Stansted site will be marginally more of a noise nuisance than the other five sites on the airport authority's short-list.

The application for development permission has gone to the Essex County Council. There will be a public inquiry on the proposals, probably early next year. That will be the second occasion on which the people of Stansted have been divided into hawks and doves.

Choosing a site for London's third airport has already taken 27 years. The chief problem has been that cost-benefit analyses yield equivocal results even when they themselves, like the Roskill Commission of 1968–70, cost more than £2 million. Uncertainty about the growth of demand for air travel has also taken the steam out of what seemed to be an urgent problem ten years ago.

According to NAC, Maplin, situated on the north of the Thames estuary, would disturb nearby residents least but would require the removal of a Ministry of Defence establishment — which might create environmental problems of its own — and the building of new road and rail links into London. It would also disrupt local bird life. Stansted, an inland site north of London, would be the cheapest site to develop — it already has a small airport, which could be extended, and good links with London, but it is situated near several small towns. The NAC report estimates that 17,000 people would find aircraft noise a nuisance if a two-runway airport were built.

Estimates of annoyance caused by aircraft noise are themselves equivocal. The Noise Advisory Council quotes values of the Noise-Number Index, calculated in such a way as to correlate with surveys of the extent to which people are annoyed by aircraft movements. One difficulty in using this index as a basis for assessing the impact of new airports is that the calibration has been based on surveys at established airports, such as Heathrow, where residents have to some degree become accustomed to aircraft noise after long exposure. Further difficulties stem from uncertainty about flight paths, the noisiness of future aircraft and the future volume of air traffic.

The NAC report says that the noise dis-

Shuttle for orbit

The US National Aeronautics and Space Administration has screwed up its courage and settled on a launching date for the first orbiting version of the space shuttle — March 1981. The decision was taken at the end of last month and in the face of the setback a few days earlier when a fire damaged a shuttle engine during a test firing in Mississippi.

The decision follows a week-long review of the recent ups and downs of the development of the shuttle, and has apparently been taken in the belief that only a tight schedule will sufficiently concentrate the minds of those involved in putting the first shuttle (called Columbia) into an Earth orbit.

The timetable now agreed would move Columbia from the building it now occupies at Cape Canaveral to the shed in which it will be mated with the solid fuel boosters on 23 November. Thereafter it will be mounted on its launch pad and the main engines of the shuttle will be fired for a period of 20 seconds some time in February. Thereafter, there will be five weeks to the final launch date.

Although anxiety persists about the tiles intended to provide thermal shielding for the shuttle, it has apparently been decided not to encumber the first flight with the "tile repair kit" intended to enable the crew of the shuttle craft to replace damaged tiles in flight.

advantage of Stansted over the other sites, excluding Maplin, is only marginal. But it recommends that if Stansted is chosen, local residents should be compensated either in the form of noise insulation or by grants to enable them to move house. It further adds that Stansted could be justified on environmental grounds only if it reduced the total environmental impact of London's airports, especially Heathrow, where about one and a half million people live within the contour of noise annoyance. Hitherto, the case for a third airport has been to absorb increases in demand for air travel rather than to take pressure off existing airports.

The airports authority's proposal for Stansted is for a one-runway airport capable of handling 15 million passengers a year — considerably smaller than a previous proposal thrown out in 1965–66 to build an airport for 100 million passengers a year. One of the additional attractions of the Stansted site is that the existing airport could be enlarged gradually to meet changes in demand.

Judy Redfearn

Polish water

Much and little

Last month's floods, which produced emergency conditions in 22 out of the 49 provinces of Poland and caused a massive loss of crops, especially hay, potatoes and sugar beet, have focused attention once again on the paradox of Poland's water supply. Ranking only twenty-second in Europe in *per capita* water index (1,700 m³ a year compared with the European average of 4,800 m³ a year), Poland is nevertheless subject to periodic inundations after which the water runs off uselessly into the Baltic. At best, Poland can store only 5 per cent of the water it receives from natural precipitation and incoming rivers. (Bulgaria, Czechoslovakia and the Soviet Union, by contrast, store 12–15 per cent.) Along Poland's largest river, the Vistula, there is no storage capacity whatsoever.

To remedy this, the Central Committee of the Polish United Workers' Party adopted on 16 June 1978 a resolution calling for a multi-disciplinary development and management programme for the Vistula. "Program-Wisla", as it is called, got off the ground very rapidly, at least on the theoretical and planning side. Polish geographers, hydrographers, economists, agriculturalists and ecologists produced imaginative plans which include hydroelectric stations, irrigation works, the improvement of navigation channels, the construction of retaining walls and some 34 or 35 dams along the navigable course of the river between Oswiecim (Auschwitz) and Gdansk. The whole scheme, which is intended to raise Poland's water storage capacity to 20 per cent, as well as benefiting agriculture, industry and

the human environment, is scheduled for completion by the end of the century.

So vast an enterprise, say the planners, is nevertheless beyond the capacity of Poland alone, and there will be major scope for international cooperation. But before the Vistula is confined by the engineers into what will be, in effect, a series of basins, the water must be of acceptable purity, since once the natural scour is eliminated, the ecologists agree that the river would not regenerate itself. The Vistula (like Poland's other major river, the Odra) has no water of class I or II purity anywhere in its course, while one-third of its total length is polluted beyond the limit of toleration. Purification and regeneration of the river is the first priority of Program-Wisla.

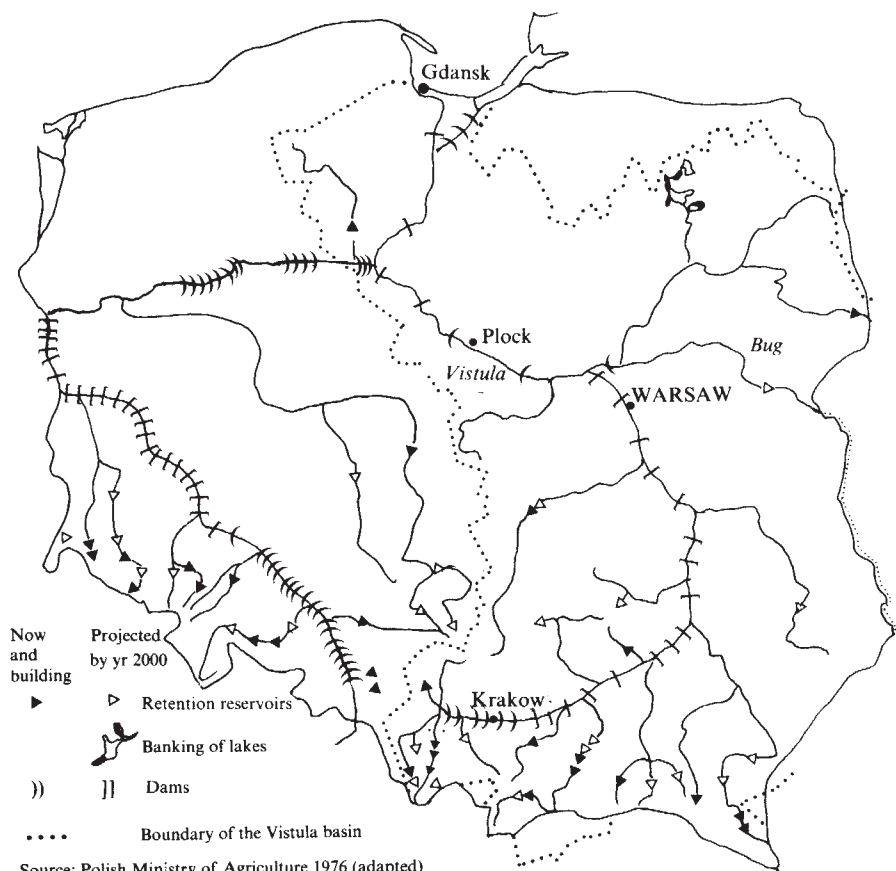
The problem, however, is far from simple. The headwaters of the Vistula are subject to natural "pollution" by mineral salts leached out of the soil of Silesia. The river flows through the Silesian industrial belt and the Cracow-Nowa Huta conurbation, with its vast steel mills, and past the open-cast sulphur mines at Tarnobrzeg. It picks up its largest tributary, the Bug, already heavily polluted by the Soviet pulp and paper industry, passes through Warsaw, whose sewerage arrangements are, to say the least, antiquated, through Plock with its mushrooming petrochemical plant and finally arrives in the Bay of Gdansk where pollution is so heavy that water-sports are prohibited.

There are nevertheless some bright spots in this picture. The Tarnobrzeg mines, in

particular, are managed on strict ecological principles, with recultivation of the land section by section as soon as the sulphur is stripped. Water control there, say the mine engineers, has to prevent seepage from the river to the mine rather than the reverse. The Bug, the one part of the Vistula basin not entirely under Polish control, has just been made the subject of a special Polish-Soviet cooperation agreement. And week after week, the Polish media stress the emphasis being given to anti-pollution measures, effluent treatment and the introduction of closed cycle technology under the aegis of Program-Wisla.

Such technology or the exchange of know-how could well be a fruitful field for international cooperation, both within and outside the Comecon bloc. But Polish experts have hitherto been cool at the prospect of Western cooperation, feeling that their prospective partners have been more interested in selling existing standard equipment than providing know-how specifically tailored to Polish circumstances. Since the Polish economy suffers from a chronic lack of hard currency, this is a major problem. The latest negotiations, between Poland and the United Kingdom, seem to be taking a different and — from the Polish point of view — a more promising course. It is understood that before any concrete proposals are put forward from the British side, a small investigative team will visit Poland in the autumn to make an on-the-spot assessment.

Vera Rich



NEWS AND VIEWS

The cooling Earth

from Henry N. Pollack

WHAT can the exhaust of the Earth's heat engine tell about its internal workings and fuel? The exhaust is the heat lost from the Earth's interior, and the internal workings are the manner by which heat is transported upward from the deep interior. The fuel is thought to be principally nuclear, comprising the radioactive isotopes present in varying trace concentrations throughout the Earth.

Let us look first at the exhaust. The measurement of the heat flux over the Earth's surface is an endeavour of the last two decades, and the global survey is still only half complete. However, an important empirical rule, that heat flow varies inversely with crustal age, has enabled estimates of the heat flow to be made for the unsurveyed areas of the globe. At the present time the Earth seems to be discharging about $40 \times 10^{12} \text{ W}$ (ref. 1-3).

Does the engine run smoothly, or does it race, or idle on occasion? How characteristic of recent Earth history is the present-day heat loss? Because the heat loss is strongly correlated with the age of the ocean floor, which in turn is related to oceanic crustal accretion rates that are known to be variable, it is likely that the heat flow has fluctuated in the past. Sprague and Pollack⁴ have reconstructed the age distribution of oceanic and continental crust over the past 180 Myr and conclude that the heat loss has ranged between 39 and $52 \times 10^{12} \text{ W}$, with an average of $43 \times 10^{12} \text{ W}$, slightly higher than the present-day value.

The internal workings of the Earth engine are only weakly constrained by knowledge of the heat loss alone. The planetary thermal history models of 20 years ago were principally conductive models, in accord with the then prevailing view of an Earth without global scale

horizontal movements. However, even before mobility became accepted in the 1970s, Tozer⁵ argued that thermally-activated solid-state creep would, in response to thermally induced buoyancy, lead to large scale mobility within the Earth. The temperature dependent creep serves as a thermostat, with convection relieving the Earth of excessive heat by transporting it to the uppermost mantle where it makes the final journey to the surface by conduction through the upper horizontal boundary layer of the convection. This upper boundary layer comprises the plates of the lithosphere.

While the concept of solid-state thermal convection in planetary interiors is clear enough, a complete mathematical description of the process is difficult. The patterns of the Earth's topography, heat flow, gravity, and plate velocities provide surface constraints, but even so the broad features of the convection remain elusive. However, simpler quantitative treatments, which yield certain general characteristics of the convection system have recently been exploited⁶⁻⁹; collectively they are referred to as 'parameterized' thermal history models. At the heart of these analyses is the assumption, suggested by both theory and experiments, that the heat loss from a convecting system depends on the temperature difference driving the convection (the so-called Nusselt number — Rayleigh number relationship). The models yield the history of such properties as the mean temperature and viscosity of the mantle and the mean velocity and radial heat flux of the convective circulation over the lifetime of the Earth. The calculations all explicitly require the specification of the variation with time of the Earth's internal heat production, and thus a link is forged to the Earth's nuclear fuel and its characteristic rate of decay.

The existence of nuclear fuel in the Earth has been known ever since the discovery of radioactivity in rocks in the early part of this century. However, the abundance and blend of the fuel has been a subject of some debate. The principal heat-producing isotopes are ^{238}U , ^{235}U , ^{232}Th and ^{40}K , with respective half-lives of 4.47, 0.70, 14.0, and 1.25 billion years. Their abundances in the crust of the Earth are comparatively well

known, and recognized to be significant enrichments over any possible primordial concentrations throughout the bulk of the Earth.

What then can be said of the primordial abundances of each of the principal isotopes? It was first pointed out in the 1950s that if the Earth had an isotopic endowment similar to that of the chondritic meteorites then the internal heat production would be just about adequate to supply the surface heat flow. However, the 'chondritic coincidence' as this near equilibrium between heat production and loss came to be known, was not without some inconsistencies as well. In particular, the relative proportions of U, Th, and K in chondrites are rather different from that in terrestrial rocks. If the primitive Earth were indeed chondritic, most of the uranium apparently now resides in the crust, while most of the potassium must be elsewhere, presumably deeper within the Earth. Wasserburg *et al.*¹⁰ proposed an alternative primordial isotopic endowment which reflected the K:U:Th ratios found in the crust of the Earth. Yet a third approach has recently been put forward by Sleep¹¹ and by Stacey¹² who use the abundance of ^{40}Ar , a decay product of ^{40}K , to estimate the K abundance of the Earth. Their calculations indicate a K:U ratio even smaller than the model of Wasserburg *et al.* The significance of the K:U ratio resides in the much longer half-life of ^{238}U (the dominant uranium isotope) compared to that of ^{40}K . An Earth thermally evolving under the influence of long lived uranium and thorium has a slow and steady release of heat, with a decrease by only a factor of two or so during its lifetime. A potassium dominated thermal evolution, such as would derive from the chondritic mix, is characterized by a more vigorous early history and an eight-fold decrease in internal heat production over the Earth's history. Thus the thermal and tectonic history of the Earth is closely tied to the blend of the radio-active fuel.

Now come the new family of parameterized thermal history models, and they tell a disquieting story, at least as far as deducing something of the Earth's primitive chemistry from the present-day heat loss. It appears that the heat loss derives not only from radiogenic heat, but also in part from 'initial' heat, that energy residual from the accretion of the planet and settling out of the metallic core. The

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calculated fraction of the present-day heat loss attributable to the initial heat appears to be highly model dependent; Schubert *et al.*⁷ estimate that at least 15-35% of the heat flow is non-radiogenic, while Sharpe and Peltier⁶ contend that it is not essential to invoke *any* radiogenic heat to understand the main thermal features of the Earth. Initial heat alone may be adequate to drive the Earth engine. Schubert *et al.* emphasize that while the amount of the non-radiogenic heat loss may be reasonably debated, a significant contribution to the total heat flow is unavoidable.

Some generalities do emerge from all the new thermal histories: 1) the Earth is and has been cooling, albeit very slowly, at a rate probably no greater than 100° C per billion years (the quintessential energy storage device!), 2) the cooling has been accompanied by a stiffening of the mantle

and a slowing of the convective circulation, 3) the upper boundary layer of the convection, that part of the circulation moving horizontally at the Earth's surface and comprising the lithospheric plates, has thickened over the Earth's history. But if through these models we have learned a little of the functioning of the Earth's engine, it seems we know less than we thought about the Earth's primitive chemistry. The engine apparently will run on nearly any blend of fuel, and the thermal energy exhausted reveals little of the internal chemistry. The principal sources of geochemical insight, alas, must continue to be the continental and oceanic crustal rocks, salted with occasional bits of the mantle, and the much smaller collection of extraterrestrial samples; less help appears to be forthcoming from the Earth's heat than a generation of earth scientists had hoped for. □

The collagen gene family

from Ellen Solomon

COLLAGEN proteins occur as a family of molecules with closely related structures but varying tissue distributions. At least nine different genes code for protein with collagens unique features of a repeating pattern (every third amino acid is glycine) and large amounts of the unusual amino acids hydroxyproline and hydroxylysine.

The total number of genes in the family may be as high as fifteen or sixteen if some of the less well characterized chains are included, as well as the A,B,C, chains of Clq, and if some of the observed heterogeneity in sequence is due to different gene loci. These genes have almost certainly evolved by duplication and divergence of a common ancestral sequence, but nothing is yet known of their linkage. cDNA and genomic clones have been isolated from the chick $\text{pro}\alpha_1(\text{I})$ chain (Lehrach *et al.* *Proc. natn. Acad. Sci. U.S.A.* **75**, 5417; 1978; Sobel *et al.* *Proc. natn. Acad. Sci. U.S.A.* **75**, 5846; 1978; Vogeli *et al.* *Nucleic Acid Res.* **8**, 1823; 1980). Based on clones spanning 55 Kb of the chick genome, de Crombrughe (National Institutes of Health) estimates that the $\text{pro}\alpha_2(\text{I})$ gene covers 40 Kb and that there are more than 50 intervening non-coding sequences that vary in size from 60-70 to 3-4,000 bp. These workers have analysed six coding regions in three separate regions of the helical portion of the molecule; five of these are 54 bp (18 amino acids). They propose that the helical portion of the collagen gene arose by duplication and divergence of a 54 bp sequence and that these regions diverged by the insertion or deletion of units of 9 bp — the basic (Gly-X-Y) repeat of collagen. Boedtker and colleagues (Harvard

University) have analysed the codon usage for the amino acids in the $\alpha_1(\text{I})$ and $\alpha_2(\text{I})$ chains of Type I collagen and find that the usage differs significantly between the chains. Genomic clones of the sheep $\text{pro}\alpha_2(\text{I})$ have also been isolated (Schafer *et al.* *Nucleic Acids Res.* **8**, 2241; 1980, and show at least 17 intervening sequences in 60% of the 3' portion of the gene. Non-coding sequences are 300 to 1,600 bp, while the coding regions are 250 bp or less. Somatic cell genetic studies using species-specific DNA probes should soon clarify the location of some of the collagen genes and their possible synteny, if not linkage.

Haemoglobins (Weissman, Yale University), histones (Zweidler, Institute for Cancer Research, Philadelphia) and heat shock proteins (McCarthy, University of California, San Francisco) were discussed as representatives of rather different types of gene families, in regard to linkage and co-ordinate expression. Until the genetic map of the collagen genes is known it is obviously not possible to know if, as a family, it resembles any of these others. By analogy with the non-linked α and β genes, and the linked $\beta \cdot \delta \cdot \gamma$ genes of haemoglobin, will the $\text{pro}\alpha_1(\text{I})$ and $\text{pro}\alpha_2(\text{I})$ genes of collagen be unlinked and the $\text{pro}\alpha_2(\text{I}, \text{II}, \text{III}, \text{IV}, \dots)$ linked? Alternatively, will all the "collagenes" form a linked cluster as with the histones, or be scattered throughout the genome as with the actin, tubulin, and heat shock genes of *Drosophila*? As yet there is no information as to whether any of the collagen genes exist in multiple copies.

There are inherited connective tissue diseases which are likely to involve defects in collagen genes, such as Marfan's Syndrome and osteogenesis imperfecta and approaches towards their understanding were discussed. These diseases have eluded

definition at the molecular level, largely because of the extreme difficulty of the protein chemistry. It was agreed that current DNA technology should be applied to defining these diseases, bearing in mind that in most cases there is no direct evidence for collagen gene involvement. It is not reasonable to clone collagen genes from individuals with connective tissue disorders without direct evidence that these genes are actually involved as the primary defect. A more general approach is needed whereby collagen gene mutations can be detected. One suggestion was to screen as many different patients as possible, with different diseases, by restriction mapping and Southern blots using collagen probes, and where appropriate tissue biopsies are available, by S1 mapping. Using these two techniques some types of mutations in the collagen genes could be detected. For results from either of these techniques to be meaningful, however, a great deal of data is needed regarding the degree of variation in these genes in the normal population. A second approach discussed at length was that of linkage analysis within families using the collagen gene probes and restriction site polymorphisms, as done by Kan and Dozy, for sickle cell anaemia (*Lancet* **ii** 910; 1978). The collagen diseases are rare, extremely heterogeneous, both clinically and genetically, and probably often represent new mutations. They are unlikely to have evolved by selection, as have some of the haemoglobinopathies, and it was therefore emphasised that each family would have to be screened with a set of restriction enzymes to find an appropriate polymorphism. The purpose of this type of analysis would be both to identify affected fetuses by prenatal diagnosis, and also to indicate the involvement of a collagen gene defect in those diseases.

Also discussed was the complexity of the genetics of a protein with as many enzymatic post-translational modifications as collagen. For example, mutations in different modification enzymes could lead to the same disease phenotype, and different mutations in the same enzyme could give dominant or recessive modes of inheritance.

The extensive post-translational modifications of collagen and proteoglycans, including removal of signal sequences, hydroxylation, glycosylation, disulfide bond formation, triple helix formation, and procollagen to collagen conversion was an important topic. Different types of collagen are modified to different extents and individual types of collagen show a degree of heterogeneity in their particular modification. Among the questions regarding modification, therefore, is to what degree individual primary amino acid sequences determine the position and extent of, say hydroxylation

A conference on 'Gene families of collagens and other proteins' was held from April 27-May 2, 1980 at CMDNJ — Rutgers Medical School. The organisers were D. J. Prockop and P. C. Champe.

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and glycosylation, and whether there are tissue-specific enzymes for each of the different types of collagen. As yet there is no direct evidence for such enzymes (Kivirikko, University of Oulu, Finland). This may well be due to the difficulty in assaying many of these enzymes. The need exists for more rapid and specific assays for each enzyme including electrophoretic separation techniques, and immunological or colourimetric detection. The questions of tissue-specific forms of the enzymes, genetic map location, possible linkage of groups of these enzymes, linkage to the collagen genes, and control of expression could be approached if less cumbersome assays could be developed.

A final major topic was the extracellular

matrix (collagen, fibronectin, proteoglycans, fibrin) and the cytoskeleton (actin, microtubules, intermediate filaments). Through much information exists on structural differences between individual members of the gene families (collagen, actin, tubulin, keratin) the functional significance of these differences is not at all clear. Certain common functional domains can, however, be identified. For example, the collagen cross-linking regions and collagenase cleavage site are strongly conserved in collagen Types I, II and III. Other macromolecules are associated with collagen fibres, and may have specific interactions with the collagen and each other. The most clearly defined system is that of cartilage proteo-

glycan, where the proteoglycan combines with hyaluronic acid and link protein to form large aggregate structures (Muir, Kennedy Institute of Rheumatology, London). It is anticipated that collagen will also be incorporated into such multimeric structures. Fibronectin has a number of separate functional domains that allow it to bind to collagen proteoglycan and the cell (Vaheri, Helsinki University). Martin (National Institutes of Health) reported that proteins analogous to fibronectin but specific for chondrocytes (chondronectin) and epithelial cell (laminin) have been discovered. These bind specifically to these cells and their collagens. Such interactions may explain the role of the tissue specific collagens.

Assembly of immunoglobulin heavy chain genes

from H. V. Molgaard

IT has been clear ever since it became possible to compare the amino acid sequences of immunoglobulin molecules that they pose two formidable problems for genetics. The first is the extreme variability of the N terminal portions (V regions) of the chains, which make up the antigen-recognition site. The second is how one of a very large number of V regions can come to be associated with one of a very small number of the constant regions (C regions) which mediate the effector functions of the molecule in the elimination of antigen.

It was the occurrence of a very variable sequence joined to a relatively constant sequence that led Dreyer and Bennet to propose that the V and C genes are separately encoded in the genome¹ — a proposal which was confirmed by the first DNA sequence studies on mouse light chains. Those studies showed that a large but limited set of V genes exists at some distance from the C gene in the germline DNA. During the maturation of an antibody-producing B cell, one of the V genes is brought into the proximity of the C gene. As it has turned out, this translocation also provides at least a partial explanation for antibody diversity, since it has proved that the V region is itself constructed from two genes: the V gene and one of four J genes adjacent to the C gene on the germline DNA. V genes seem able to combine at random with one of the J genes, the exact site of the junction varying slightly and coinciding with hypervariable region 3 on the light chain².

DNA sequence analysis has now been extended to the mouse heavy chain genes, which are of particular interest because of the evidence that each V gene may be translocated more than once in the course of ontogeny. Unlike the light chains, for which there is only one C region gene for

each set of V regions, each heavy chain V gene (V_H gene) may be expressed with any of eight different heavy chain constant genes (C_H genes), C_μ , C_γ , $C_{\gamma 1a}$, $C_{\gamma 2b}$, $C_{\gamma 3}$, C_α , C_ϵ , C_δ , which define the heavy chains as μ , γ , α etc and the different classes of antibody molecule, IgM, IgG, and so on. All V_H regions are expressed first in μ chains, but the same V_H sequence may later be expressed as part of a γ chain or an α or an ϵ chain. The mechanism of this phenomenon, the heavy chain switch is therefore a mystery peculiar to the heavy chain genes. DNA sequencing has shed some important light on this mystery; but it has also shown that the V regions of the heavy chain genes have a more complex structure than their light-chain partners.

The V_H region is encoded by three genes.

Hybridisation of germ line DNA with V_H probes has shown that there are at least 50 and probably many more V_H genes, arranged in tandem arrays³⁻⁵ and separated by an unknown length of DNA from a cluster of C genes⁶.

Comparison between the DNA sequence of a germ line V_H gene and the corresponding rearranged sequence expressed in a myeloma have in two different cases — a V sequence expressed in an α chain⁷ and another expressed in a γ_{2b} chain⁸ — shown that the germ line V_H segment ends at the sequence coding for the third hypervariable region before the end of the expressed V_H sequence. This suggested, by analogy with the light chain genes, that the missing sequences should be found in J segments near a C gene. Since C_μ is the first C gene to be expressed in B-cell ontogeny, the J segments were sought and found near the C_μ gene in a cluster of four, 8kb upstream from C_μ . However, the J segments still do not

account for all of the third hypervariable region. This has led to the suggestion that the missing residues are separately encoded in yet another set of gene segments, the 'D' or 'diversity' segments, the existence of which had already been predicted on the basis of the amino acid sequence data^{9,10}.

It remains controversial whether heavy chain diversity is fully accounted for by random combination of V, D and J segments and codon variation at the joins of these segments or whether further diversity arises out of point mutations at the other two hypervariable regions during the differentiation of the B lymphocyte.

Mechanism of V gene rearrangement

With the availability of data from both light and heavy chain germ line gene segments it is possible to generalise about the mechanism of V-J segment translocation. In both cases it involves deletion of the intervening DNA^{4,11} and in both cases two short conserved sequences occur in the sequences 3' to the V segment and 5' to the J segment close to the point of recombination.

At the germ line V_H and J_H segments which are rearranged and expressed in the S107 myeloma the two conserved sequences are arranged⁷ as shown below. One strand is shown folded back on itself to illustrate that the J_H segment conserved sequences are almost inverse complements of the V_H segment sequences. Comparison of these sequences at all the known light and heavy chain V segments shows that they can all be derived from the heptamer CACAGTG and the nonamer ACAAAAACC with usually only one or two base changes. Similarly the conserved sequences at all the J segments derive from CACTGTG and GGTTTTGT.

Another striking feature of these

	residue 101 Asp	non coding sequences
5' V_H segment . . .	GAT	GCACACAGTG — 23 nucleotides — ACACAAACC
3' J_H segment . . .	CAT	C GTGTCAG — 22 nucleotides — TGATTTTGA
	residue 107 Tyr	non coding sequences

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conserved sequences is that the length of the random sequence separating the heptamer and nonamer is highly conserved. At the heavy chain V and J segments the separating sequences vary from 21 to 24 base pairs (bp) in length. In contrast, in the two light chain families, the κ and λ chains, the spacers at the V segments differ from those at the J segments. The spacers at the V_{κ} segments are 11 or 12 bp long while the spacers at the J_{κ} segments are 21-24 bp long. Conversely at the V_{λ} segments the spacer is 22 or 23 bp while at the J_{λ} segments a 12 bp spacer occurs.

Spacer lengths of 11-12 and 21-24 bp correspond closely to one and two turns of the double helix, and mean that the heptamer and nonamer form a nearly continuous sequence around the double helix interrupted by one or two complete turns. Since in light chain V-J recombination, a one turn spaced heptamer and nonamer and a conserved sequence interrupted by two turns occur at the two recombining segments Early *et al.*⁷ and Sakano *et al.*⁸ propose that two different but closely related joining proteins recognise the one and two turn spaced sequences and that these proteins form a complex within which the cutting and joining of the DNA takes place. As it is likely that the heavy chain V and J segments recombine first with a D segment this model predicts that the D segments should be flanked by these conserved sequences interrupted by one turn. It would also rule out recombination within the V and J segments. Early *et al.*⁷ argue against a mechanism involving the previously proposed stem structures^{11,12} which can be formed between these inverse complementary sequences flanking the V

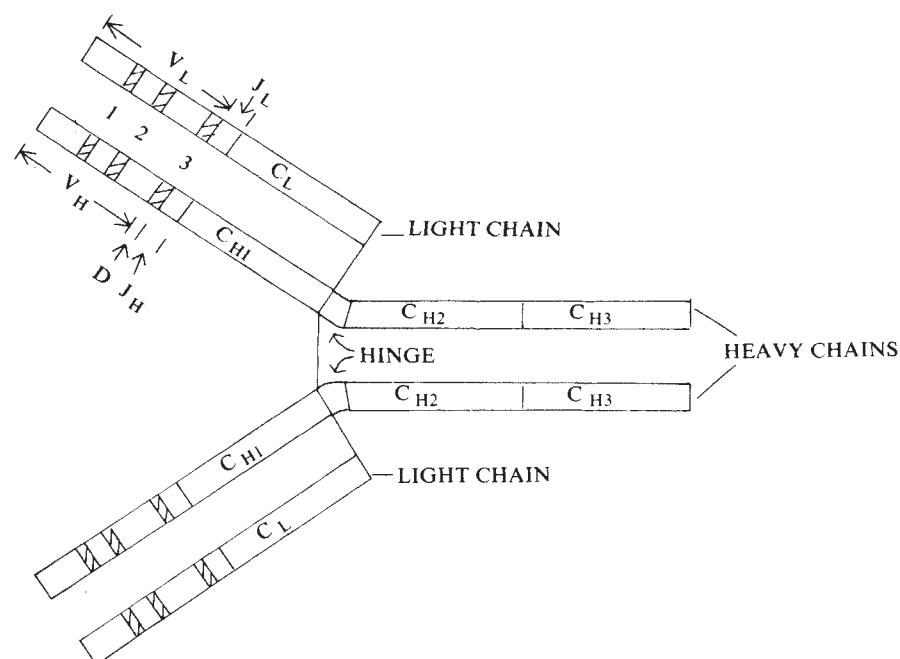


Fig. 1 Schematic diagram of an IgG antibody molecule. C_{H1} , C_{H2} , C_{H3} and C_L are the heavy (H) and light (L) chain constant region domains — these are regions 100-125 amino acids long within the chains which show some homology with each other in amino acid sequence and secondary structure. The parts of the light chain V region coded by the V_L and J_L gene segments are shown as are the sections of the heavy chain V region coded by the V_H , D and J_H gene segments. The hypervariable regions 1,2,3 determine the antigen recognition and binding site. The C regions mediate the effector functions involved in the elimination of antigen.

and J segments on the grounds that such structures are unlikely to form spontaneously between such widely separated segments of DNA and that they do not account for the conservation of the spacer sequences.

These sequence homologies between the light and heavy chains suggest that, whatever the recombination mechanism, it is the same for both light and heavy chain genes. As the two light chain families and the heavy chain family are each on different chromosomes recombination between families is prevented.

The mechanism of the heavy chain class switch

A further important implication of the germ line organisation of the heavy chain genes can be drawn from the discovery that the J segments which are expressed in α and γ_{2b} heavy chains of myeloma proteins are next to the C_{μ} gene in the germ line: this establishes that the translocation of V_H and C_H gene segments to give complete α and γ_{2b} chain genes must have been preceded by the recombination of a V_H segment with a J_H segment at the C_{μ} gene. The second recombination event, the heavy chain switch, occurs between a complete $V_H J_H C_{\mu}$ gene and, for example, either a C_{δ} or a C_{γ_2} gene which is still in a germ line context. In addition to the recombined $V_H D J_H$ segments, a considerable portion of the $J_H C_{\mu}$ intron is translocated, giving a new intron in the expressed α or γ gene whose sequences originate partly from the $J_H C_{\mu}$ intron^{13,14,6 & 8}.

In the hope of identifying specific signals

in the DNA sequence which could mediate the heavy chain switch, Sakano *et al.*⁸ have identified the sites in the $J_H C_{\mu}$ intron and in the sequences 5' to the germ line $C_{\gamma_{2b}}$ gene where the recombination event which gave a complete expressed γ_{2b} gene had occurred, and have sequenced the DNA around these sites. Two short sequences, TCCTGG and AGA, occur at equivalent positions 5' to the two sites and could be the signals for the μ to γ_{2b} switch. However, these sequences do not occur at the μ to α^8 and μ to γ_1 ¹³ switch sites, both of which lie in a region of tandemly repeated sequences in the $J_H C_{\mu}$ intron. It seems that the switch sites are different for the different class switches and may even vary within one type of class switch¹⁵. The recombination sites involved in the class switch need not be unique as long as they do not create signals which terminate transcription or destroy the signals required for the splicing event which removes the intron containing these sites.

It is possible that the homologies in the flanking sequences of the different C_H genes are a consequence of their evolutionary history. Each C region consists of three or four (depending on the heavy chain class) partly homologous domains thought to have evolved by tandem duplication from a common sequence coding for a single domain. Homologies in the flanking sequences might then have been conserved if they had come to acquire a role in the recombination events leading to class switches. If indeed the three domains of each C region derive from the same gene, then weaker sequence homologies might be expected in the

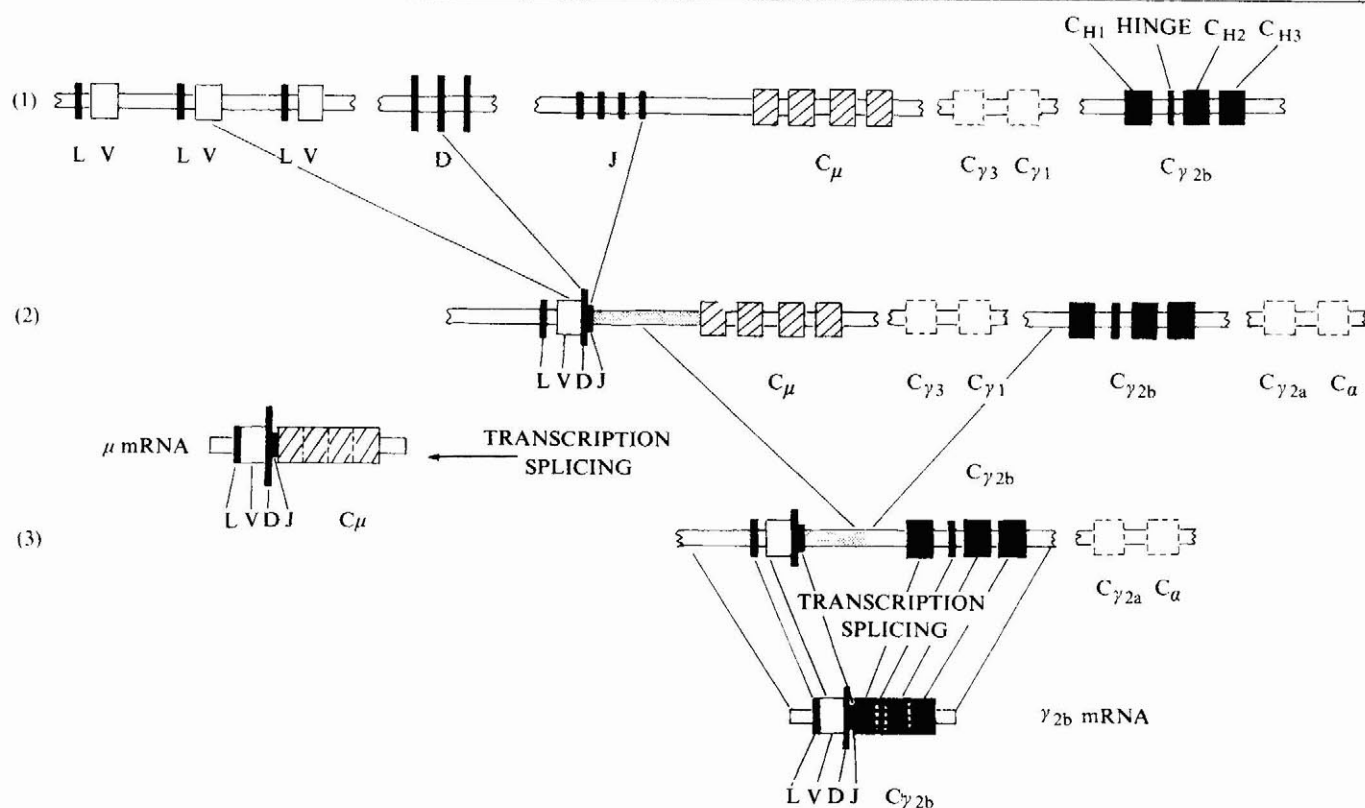


Fig. 2 Recombination events leading to a complete γ_{2b} gene. (1) The germ line DNA before rearrangement. These is a cluster of at least 50 genes (each with a short leader sequence L) coding for part of the variable (V) region, a cluster of D gene segments coding for most of the third hypervariable region and some distance away there are 4 J segments which complete the V region coding sequence. The J segments lie about 8kb from the C_μ gene which lies at the start of a cluster containing all the C region genes. The C region gene sequences are interrupted by non-coding sequences to give a series of exons which coincide with the domains and the hinge region in the C region amino acid sequence. (2) In the first translocation event one of each of the V, D and J segments are recombined to give a complete μ chain transcription unit. The transcript is a copy of the gene as shown but the non-coding sequences (introns) are removed by splicing events which lead to a continuous coding sequence in the μ m-RNA. (3) A second translocation event, the heavy chain switch, deletes the C_μ , $C_{\gamma 3}$ and $C_{\gamma 1}$ gene segments and places the VDJ segment and part of the J- C_μ intron near the $C_{\gamma 2b}$ gene. Following transcription the introns are removed by splicing leading to a continuous coding sequence in the γ_{2b} mRNA.

intervening sequences that have been found between constant region domains. Such homologies have in fact been found in an investigation of a deletion mutant in a myeloma cell line. An illegitimate recombination event or class switch has led in this case to the deletion of an exon coding for the first domain in $C_{\gamma 1}$ (ref. 16). That the region of tandemly repeated sequences in and near the switch region is a particularly good site for recombination is illustrated by the spontaneous deletions which occur in this region, on cloning or propagation in *E. Coli* and which have prevented several groups from isolating complete DNA fragments containing these C_μ flanking sequences^{8,14}.

The heavy chain switch involves deletion of C_H gene segments.

The overwhelming evidence of a number of experiments by different groups in which cloned constant region probes have been used to probe the DNA of many different plasmacytomas is that the expression of a constant region class other than μ involves deletion of other constant region genes including C_μ in a way which is consistent with a C_H gene order $\mu-\gamma_3-\gamma_1-\gamma_{2b}-\gamma_{2a}-\alpha$ (δ and ϵ have not yet been studied) and a model in which the genes preceding the expressed gene are deleted^{4,16-18}. Some controversy remains however as to whether

the deletions occur only on one chromosome or on both. Honjo and Kataoka¹⁹, who were the first to find evidence for the deletion model, concluded on the basis of DNA hybridization kinetics that deletion was confined to one allele. Yaoita and Honjo²⁰ have now produced stronger evidence for deletion on only one allele by analysing a IgG_{2b} secreting myeloma induced in an F₁ hybrid of C57BL and BALB/c mice. A judicious choice of restriction enzymes enabled them to distinguish the two alleles and to show that the C_μ and $C_{\gamma 1}$ genes of the expressed allele are deleted and the expressed $C_{\gamma_{2b}}$ gene is rearranged, while the unexpressed alleles C_μ , $C_{\gamma 1}$ and $C_{\gamma_{2b}}$ are unchanged. They did find two unexpected rearrangements in the 5' flanking sequences of the unexpressed C_μ allele and in the 3' flanking sequences of the unexpressed $C_{\gamma 1}$ gene.

However, in the numerous myelomas analysed by the other groups deletions and rearrangements often occur on both alleles. Unexpected rearrangements such as the rearrangement of both C_μ alleles in some IgG_{2a} expressing tumours has led Coleclough *et al.*¹⁷ to propose a stochastic model in which, after the first translocation which creates the complete μ gene, deletion events may occur within the C_H cluster on both alleles, only some of which expose a

V_H gene to a second C_H gene leading to a heavy chain switch. Other deletions may have no effect on expression or may limit the options for further switching.

It should be borne in mind that all these studies have been carried out with mouse plasmacytomas which are known to show chromosomal abnormalities, are of unknown ploidy, being perhaps mostly hypo tetraploid²¹ and have been maintained in culture much longer than the life time of a normal diploid B-cell. Some of the events observed in the myelomas may not occur or may be infrequent in normal cells. In an isolated study of a mixed population of normal B cells Joho and Weissman²² produced evidence consistent with rearrangement occurring on only one C_μ allele. The question has been raised, however, of whether the technique they used has sufficient resolution to detect cells with both chromosomes rearranged if those cells are in a minority. The events on the second allele are important as only one allele is expressed (allelic exclusion).

With the question of allelic exclusion, molecular immunologists are drawn into the issue of how events occurring on one chromosome might affect those occurring on another; and thus into fields of enquiry even thornier than those they have so successfully tackled in the last five years. □

The origins of marine bioluminescence

from Frank McCapra and Russell Hart

THE astonishment of seeing bright light issuing from living organisms is sometimes reflected in the view that the light must have a unique biochemical origin. Yet a timely reminder of the wide variety of the chemical systems involved has been provided by the recent observations of Dunlap, Hastings and Shimomura (*Proc. natn. Acad. Sci. U.S.A.* **77**, 1394; 1980). They have demonstrated that although two distantly related organisms, the dinoflagellate *Pyrocystis lunula* and the euphausiid shrimp *Meganycitiphanes norvegica* have the same novel luminescent reactant or luciferin this molecule has no structural similarity to the luciferins identified in more than 20 other organisms.

The luminescent reaction in most organisms occurs by the action of a luciferase upon a luciferin. The similarities in the luciferins and luciferases in different organisms can be traced both by seeing if a light emitting cross-reaction is possible between the chemicals extracted from each of them and by elucidating the chemical structure of the luciferins themselves.

Experiments of this type have shown that although there is no simple pattern of distribution of particular luciferins and luciferases among the light emitting organisms one molecule does appear again and again. This 'coelenterate-type' luciferin (luciferin 1, see Fig. 1) either in the free state, protein-bound or as a simple derivative, has been found in a variety of coelenterates and in such unrelated animals as shrimp, squid and fish (see Table 1). This widespread distribution may best be explained if the luciferin derives from a common origin in the food chain.

A second luciferin (luciferin 2, see Fig. 1), 'Cypridina luciferin' has a similar structure to coelenterate luciferin sharing the same imidazopyrazine nucleus and producing light by an identical chemical mechanism (McCapra *Accounts Chem. Res.* **9**, 206; 1976) but it has no amino acids in common with luciferin 1 and must have a separate origin. No single food source could be responsible for the appearance of both luciferins in higher organisms. Luciferin 2 is also widely distributed in a variety of organisms (see Table 2), again

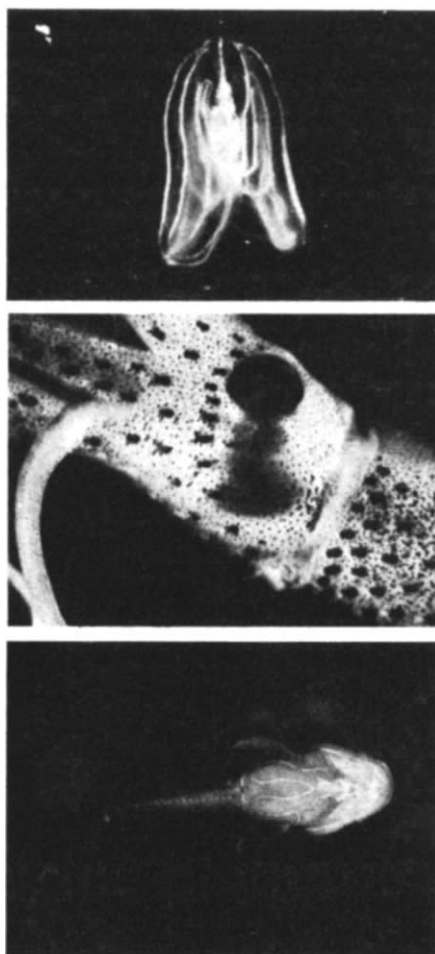


Fig. 2 *Mnemiopsis*: A widely distributed luminescent Ctenophore emitting bright flashes on stimulation; *Histiotethys*: A deep-sea squid with many complex photophores on the ventral surface and tips of the tentacles; *Porichthys*: A benthic fish found off the coast of North and South America. The many ventral photophores are used in courtship.

suggesting a food chain origin. This is supported by the finding that several luminescent fish, e.g. *Apogon* and *Parapriacanthus* (Johnson *et al.* *Proc. natn. Acad. Sci. U.S.A.* **47**, 486; 1961) have been caught with large numbers of *Cypridina* in the gut and the luciferin of these predators is identical to that of the food organism.

More direct evidence that the *Cypridina* luciferin is derived from *Cypridina* comes from the work done by Tsuji and his collaborators (*Mol. Cell. Biochem.*, **9**, 3; 1975) with the midshipman fish *Porichthys notatus* collected in two separate locations off the western coast of the US. The fish collected in Puget Sound are 'dark' although possessing the large number of highly developed photophores of the southern (Californian) luminescent fish. The only way in which luminescence could be induced in the 'dark' fish was either by injection or ingestion of *Cypridina* luciferin, demonstrating a direct link between food source and light emission.

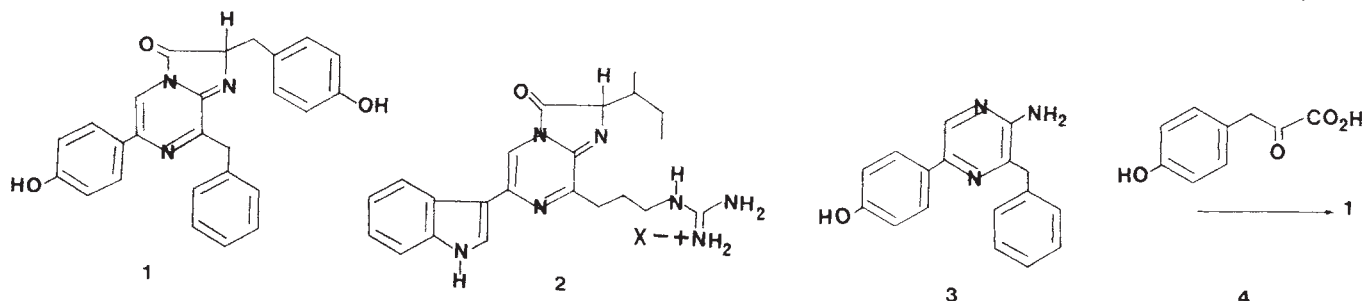
It may be that the Puget Sound area merely lacks the required, as yet unidentified, food organism containing the luciferin or a precursor. The complexity of the photophores, the nature of the transport involved and the response to administered luciferin nevertheless suggest caution in accepting this simple view. For example it is extremely unlikely that the ingested enzyme (luciferase) could survive proteolysis in the gut. In addition the endogenous luciferase in either the dark or luminous fish differs in several significant aspects from *Cypridina* luciferase.

Doubts about a food source as the sole origin of luciferin do indeed arise. For example luciferin is notoriously unstable with a lifetime in solution of a few days at most. Yet *Porichthys* luminescence, once developed by administration of small amounts of luciferin persists for up to seven months. It should be noted however that coelenterate-type luciferin is often found as a sulphate derivative, which is considerably more stable (Hori, Nakano & Cormier *Biochim. Biophys. Acta* **256**, 638; 1972). If a similar derivative exists for *Cypridina* luciferin then this would reduce the force of this objection.

Other luminous organisms (Table 3) have luciferins and bioluminescent systems with nothing in common with each other or the coelenterates.

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Fig. 1 (1) Coelenterate-type luciferin, (2) *Cypridina* luciferin, (3) amino pyrazine which can condense with (4) p-hydroxyphenylpyruvic acid to produce (1).



There is no doubt that bioluminescence arose anew many times in evolutionary history. This is shown by the markedly different structures found for the luciferins. The ubiquity of coelenterate-type luciferin does suggest a single source in this case, but may obscure several instances of *de novo* synthesis. No direct experiments have been done to settle the matter, nor do we know very much about the genetic mechanisms controlling the generation of luciferin and luciferase.

Some problems are already raised by the need to explain (in genetic terms) the simultaneous appearance of luciferin and luciferase in the same organism. These will be compounded if it is necessary to account for their separate appearance in unrelated organisms.

A possibility is that food sources contain peptide material capable of elaboration by the luminescent organisms. Such sources may or may not be luminescent. By analogy with the firefly, it may be that degradation

of the product of the reaction or of the less stable luciferin gives rise to the amino pyrazine which may then condense with p-hydroxyphenylpyruvic acid (see Fig. 1) following a known synthesis. It is clear that no single origin can be demonstrated, and establishing the actual blend of mechanisms will require both direct feeding experiments using labelled precursors, and some insight into the genetic mechanisms controlling the whole phenomenon. □

Table 1: Organisms with Coelenterate-type luciferin 1

Anthozoa	
Sea pansy	<i>Renilla</i> spp. ^a
Sea pen	<i>Ptilosarcus guernei</i> ^a
	<i>Stylatula elongata</i> ^a
Sea cactus	<i>Cavernularia obesa</i> ^a
	<i>Acanthoptilum gracile</i> ^a
Hydrozoa	
Jellyfish	<i>Aequorea forskalea</i> ^a
Hydroid	<i>Obelia geniculata</i> ^a
Ctenophore	
	<i>Mnemiopsis leidyi</i> ^a
	<i>Beroe</i> sp. ^a
Mollusca	
Teuthoidean squid	<i>Watasenia scintillans</i> ^b
	<i>Eucleoteuthis luminosa</i> ^b
	<i>Chiroteuthis imperator</i> ^b
	<i>Onychoteuthis banksi borealijaponica</i> ^b
Sepioidean squid	<i>Sepiolina nipponensis</i> ^b
Pisces	
Myctophid fish	<i>Diaphus</i> spp. ^b
	<i>Neoscopelus microchir</i> ^b
Stomiatoidean fish	<i>Argyropelecus hemigymnus</i> ^b
	<i>Yarella illustris</i> ^b
Crustacea	
Decapod shrimp	<i>Acanthephyra</i> spp. ^a
	<i>Oplophorus spinosus</i> ^b
	<i>Heterocarpus</i> spp. ^b
	<i>Systellaspis</i> spp. ^b
	<i>Sergestes</i> spp. ^b
Mysidacean shrimp	<i>Gnathophausia</i> spp. ^b
Copepod	<i>Pleuromamma piseckii</i> ^c

(a) After Cormier in *Bioluminescence in Action* p. 75 (ed. Herring (Academic Press, London, 1978))
 (b) After Shimomura, Inoue, Johnson & Haneda (*Comp. Biochem. Physiol.* 65B, 435; 1980).
 (c) Hart, Herring & McCapra.

Table 2: Organisms with Cypridina luciferin 2

Ostracod crustacean	<i>Cypridina</i> spp.
Teleost fish	<i>Porichthys notatus</i>
Cardinal fish	<i>Apogon ellioti</i>
Sweeper fish	<i>Parapriacanthus ransonneti</i>

Table 3: Organisms with other luciferins

Bacteria	<i>Photobacterium phosphoreum</i>
Dinoflagellate	<i>Pyrocystis lunula</i>
Euphausiid shrimp	<i>Meganctiphanes norvegica</i>
Boring Clam	<i>Pholas dactylus</i>

A new dimension for particle detectors

from David J. Miller

THE trouble with modern particle physics experiments is that you need to detect all kinds of particles at the same time. Not so long ago there were groups of ten or so physicists who did important programmes of research with nothing much more than a dozen neutron counters, or five spark chambers and a magnet. But storage-ring physics is different. As Cashmore (Oxford) told us on the first day of the Uppsala meeting*, the PETRA e^+e^- collider at Hamburg only produces 20 collisions per intersection region per day with strongly interacting particles (hadrons) in the final state. At such rates each event must be made to give up every scrap of information that can be measured. We don't want to see just the charged tracks. We want to see which of them are pions, kaons, protons, muons or electrons. All of the γ -rays must be converted. Neutrons and long lived K^0 must be made to interact. Neutrinos will escape, of course, but it is very important that they should be the only particles to escape so that their missing energy and momentum can be inferred from the measured particles.

The new director designate of CERN, Schopper (Hamburg), told us about the plans to build a first stage 'stripped down' LEP (large electron-positron) machine in Geneva by 1986. If this goes ahead, the first proposals for detectors will be asked for in 1981.

The detectors now working at other storage-ring machines provide prototypes for many of the ideas presented at the meeting. They are constructed like Russian babuschka dolls. Around the beam-pipe is nested a sequence of different devices, each fitted tightly over the next with the minimum of lost volume and without any chinks for particles to escape. Closest to the pipe there is often a multiwire proportional chamber and usually a cylinder of scintillation-counters. These establish the position of charged tracks coming out into the detector and, even more important,

they establish the starting time. The time of flight of a particle from the pipe to an outer ring of counters, one or two metres away, is measurably different for pions, kaons and protons below about 2 GeV/c. At present, times can be measured to about 200 picoseconds, though considerably better particle identification could be made if 50 picosecond resolution were possible. Schemes for doing this were presented, but it was not obvious that any marked improvement can be made which will be as reliable, compact and robust as scintillators. Strand (Brookhaven, US) reported an elegant technique for making hodoscopes of 1 mm scintillating fibres, though this offers the advantage of good resolution at high particle fluxes rather than an improvement in timing.

The next shell of the detector is usually a tracking-chamber. At LEP it will have to be able to resolve up to 50 charged particles, many of them bunched in 'jets'. The present chambers working at PETRA (Hamburg) seem only just good enough for the job, though one of them, in the JADE detector, is more ambitious than the others. It uses fewer drift-cells and lets the electrons from a track drift further than in the conventional chambers. This, or the even more ambitious TPC (the time-projection-chamber at Stanford, built by a Berkeley group and reported at the meeting by Nygren), seemed to be the model people were taking for the LEP detector. Position resolution of 100 microns should be possible — though present detectors only achieve around 200 microns — and tracks only 5 mm apart will need to be resolved. Separating close tracks is where TPC and JADE win over the conventional chambers. But these two 'pictorial drift chamber' techniques also give many more samplings of the ionisation on a track and that can also be used to provide π/K /proton identification below 1.5 GeV/c and, in principle, from 3.5 to about 40 GeV/c by measuring the 'relativistic rise' in ionisation. There was more controversy over the measurement of the

*An international meeting on experimentation at LEP was held in Uppsala, Sweden from the 16th to the 20th of June 1980.

relativistic rise than over any other topic. Allison (Oxford) claims that the ISIS device is now achieving the predicted resolution in ionisation measurement, but there were many others who have made similar measurements and, as Lehrs (CERN) has observed, can only come within a factor of $\sqrt{2}$ of the goal. Such a factor is crucial when the effect is only a 10% change in energy-loss and one expects around $\pm 4\%$ uncertainty in measurements.

An alternative to relativistic-rise measurements is to use Čerenkov radiation to separate π , K and proton. TASSO at PETRA, as described by Leconte, has two large slices of Čerenkov counters which penetrate through the outer part of the detector and cover 20% of 4π solid-angle. There is a layer of aerogel counters with refractive index 1.023 and outside it two layers of atmospheric-pressure gas counters. This is an impressive and complex piece of instrumentation, yet it still leaves gaps — momenta at which π , K and proton cannot be separated. A recurrent call from speakers was for even lighter aerogel — a gossamer-like clear plastic — and groups at Saclay and Lund claimed that they will get the refractive index of aerogel down to 1.006 well before LEP is built. The disadvantage of the Čerenkov approach to particle identification is that it seriously impedes the other kinds of measurement which, in a general purpose detector, come outside the tracking chamber. A radical idea from Ypsilantis (Ecole Polytechnique) may overcome this by using concentric spherical Čerenkov detectors in which particles would produce ring images whose size would give the particle type and whose position would give the direction. Successful tests have been made on the detection of Čerenkov light by large-area gas counters, but a list of formidable technical problems still has to be solved before a ring-imaging central detector can be proposed for LEP.

In the central region only charged tracks are measured. But a great deal of energy goes into the neutrals; π^0 s and γ -rays or neutrons and long-lived K's. To catch the neutrals 'calorimeters' are used. These are dense arrays of heavy material and counters in which a fraction of the energy from an interacting neutral particle is converted into analogue signals which are summed to give a measurement of the energy. For electromagnetic showers the requirement is a high value of the atomic number Z, since γ -ray conversion and electron bremsstrahlung are proportional to Z^2 . The most precise energy measurements are those made with sodium iodide crystals and a number of specialised detectors ('crystal balls') have been used for γ -ray spectroscopy with a small tracking chamber surrounded by hundreds

of closely packed crystals. One such device may be needed at LEP because there are a number of very important single- γ decays which should be looked for, such as $e^+e^- \rightarrow (\text{Higgs} + \gamma)$ at the Z^0 peak, or the cascading γ decays of the "toponium" family — an expected heavy analogue of the well-established ψ/J "charmonium" family in which the $\psi(3.7) \rightarrow \gamma\chi$ processes have given useful information on quark binding. Less-specialised electromagnetic calorimeters will need similar energy resolution to the present lead plus liquid argon or lead scintillator systems, and better spatial resolution than all but the best of present detectors. Lots of new ideas for electromagnetic calorimetry were presented, including a plea by the author for the merits of solid argon, based on work by an École Polytechnique-UCL collaboration. The new suggestion which was best received was the 'Time Projection Quantameter' — a drift-chamber filled with perforated lead sheets. It could not — nor could other gas-filled calorimeters — match the energy-precision of lead-glass or lead plus argon but it has good spatial resolution and may be easier to run than some of the other devices. For neutrons and long-lived kaons a much thicker and coarser calorimeter is needed because the absorption length for strongly interacting particles (hadrons) is much longer than for electrons and γ -rays. Some of the present detectors incorporate hadron calorimeters outside the electromagnetic calorimeter and there was considerable discussion of these techniques. The best material to use is probably uranium, which amplifies and feeds back the energy normally lost as slow neutrons, but in many cases people are happy to use the segmented iron return yoke of the track-chamber magnet. It was surprising to many of us that with a conventional solenoid around the central detector and with a hadron calorimeter of uranium plus scintillator, charged hadron energies above 10 GeV/c might be better measured in the calorimeter than in the tracking chamber.

Solenoid magnets remain popular. There is a strong school of thought which advocates conventional, large-radius, low-field solenoids, rather than small superconducting ones. For specialised detectors the 'open solenoid', as used by the DELCO detector at Stanford, can allow particles out into Čerenkov counters without

interacting, but experience with the PETRA detectors shows that one radiation-length of coil does not interfere too badly with the performance of a subsequent electromagnetic calorimeter.

One intriguing development reported at the meeting may turn out to be important in many other fields as well as in high energy physics. Kunz (Stanford University) described his 'emulating processor', a small cheap home-made microprocessor which has similar internal architecture to Stanford's large IBM computer. This allows it to execute programs which have been compiled on the IBM and so take away a large part of the 'number crunching' load from the central laboratory machine. One Stanford experiment now claims to have more computing power than two or three of the major international laboratories put together. Among other powerful electronic techniques which can now be considered there was considerable interest in 'flash analogue to digital converters' — devices developed for television purposes which sample an analogue level at a rate of more than 40 MHz. They are expensive at the moment and, as an alternative, the TPC group made a strong defence of their charge-coupled shift-register technique for doing the same kind of job. They claim it costs only \$30 per channel including the analogue to digital converter. When the data has been digitised it must be checked. Do all these 'hits' constitute a track? How much energy did the calorimeter catch? It may be that the advances in hard-wired or field-programmable microprocessor logic will play a larger part in improvements of the instrumentation at LEP, compared with PETRA, than any advances in the detectors themselves. LEP will have 25 microseconds between bunch-crossings so it will be possible, according to Waloschek (Hamburg), to apply $\sim 50,000$ separate series and parallel tests on the data from one event before deciding whether to record it; and if there is a bank of emulating processors standing by it could then be possible to run through the complete analysis on the spot. A totally different approach to decision-making logic was suggested by Julia Thompson (Pittsburg) who has been experimenting with a holographic technique which might go well with fibre-optic data transmission.

Techniques already exist for doing many of the things that need doing at LEP and there is a community of high energy physicists from all over the world who are eager to get started. The teams are likely to be of a hundred or so per experiment. There will not be much that a ten-man-team could do by themselves, but since the equipment is so modular it is clear that small groups from many separate institutions will contribute their own pieces to the whole and, with the spread of remote-access computer systems, much of the analysis will still be done in universities hundreds of miles away from LEP itself. $\square$

100 years ago

In a recent paper to the Belgian Academy (*Bull.*, No. 5) Abbé Spée contends that the spectral line D3, with wave-length about 588, observed in the chromosphere and protuberances, and assigned to a hypothetical body, helium, which some suppose to have a still more simple molecular constitution than hydrogen, probably belongs in reality to this gas. As to its non-reversibility, he considers that at a very small distance from the chromosphere the solar hydrogen may be so far cooled as to be comparable to that which we manipulate, and so, unable to extinguish waves which it can no longer produce, just as a stretched cord loses the property of vibrating in sympathy if its tension have been altered.

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REVIEW ARTICLE

The ontogeny of the neural crest in avian embryo chimaeras

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The migration and subsequent development of autonomic ganglion cell precursors can be followed in suitably constructed chimaeric quail-chick embryos, thanks to the distinctive structures of quail and chick interphase nuclei. The decisive role of environmental factors arising from non-neuronal tissues on the chemical differentiation of neurones was demonstrated together with the lability of their phenotype during ontogeny.

EMBRYONIC development involves extensive movements of cells to achieve histogenesis and modelling of the body. With a few exceptions, such movements are extremely difficult to follow precisely by classical embryological methods. In fixed tissues, migrating cells, if they can be identified at all, can usually only be recognized in favourable situations, as for example when they are just leaving their site of origin. Since the expression of a particular differentiated phenotype generally occurs only after cell migration has taken place, the cells rapidly become indistinguishable from the equally undifferentiated surrounding tissues. The construction of embryonic 'fate maps' thus involves marking presumptive migrating cells so that their movements can be followed during ontogeny. This type of study, from which fundamental data on the formation and the fate of the germ layers have been obtained^{1,2}, entails differential labelling of relatively large tissue areas with rather crude methods, such as the application of vital dyes or small marking particles to specified embryonic regions. To investigate the migratory behaviour of individual cells, more precise means had to be found. One important way of achieving such refinement has been the use of techniques for combining early embryonic cells of different genotype. Such chimaeras develop normally and can be used to trace cell lineages through characteristic genetic markers.

In recent years, cell migration, cell derivation and cell-cell interactions during ontogeny of the avian embryo have become better understood, through the development of such a marking technique based on differences of the nuclear structure in two species of birds—the chick (*Gallus gallus*) and the quail (*Coturnix coturnix japonica*).

The quail-chick marking technique

The presence in all embryonic and adult cell types of the quail of a large amount of heterochromatin associated with the nucleolus was first described in 1969, and this characteristic was proposed as a cell-marking technique in combinations of embryonic cells from the quail and the chick³. Taxonomically, the two birds are closely related, but they differ significantly in the duration of their incubation period (16 days for the quail, 21 days for the chick) and their size at birth: the newly hatched chick weighs about three times as much as the quail. However, as shown by the tables of development of the two species^{4,5}, the chronology of development and also the size of the embryo differ only slightly during the first half of the incubation period, when the most decisive events of embryogenesis occur. Quail and chick cells can readily be recognized in sectioned material with the Feulgen-Rossenbeck stain or in the electron microscope after the routine uranyl acetate-lead citrate staining procedure. In undifferentiated cells, characterized by a high proliferation rate, the nucleolus is enlarged and made up of intimately associated

DNA- and RNA-containing structures⁶. In most differentiated cell types, the nucleolus is formed of a compact mass (sometimes 2 or 3) of heterochromatin, distinct from the nucleolar RNP, which occur as 'satellites' in contact with the heterochromatin. The structure of the nucleolus thus varies somewhat according to the cell type considered and the degree of differentiation reached. Nevertheless, the differences in their structures in quail and chicken cells are always conspicuous enough to make them easy to identify⁶.

Quail-chick chimaeras can be constructed either *in vivo* or in culture. In the latter case, interactions between cell types from each species can be analysed in the course of a definite histogenetic process. Organotypic culture *in vitro* or grafting of the combined tissues on the avian chorioallantoic membrane (CAM) provides suitable conditions for these kinds of experiment^{7,8}. The quail-chick system has also been applied to cell culture for recognition of nuclei in heterokaryons after acridine-orange staining⁹. However the domain in which it has so far been the most profitable is in the analysis of chimaeric embryos constructed *in ovo* by implanting quail cells into chick, or vice versa.

This method has been applied in my laboratory to study the ontogeny of two systems that involve extensive cell migrations in the embryo, the neural crest and the haemopoietic organs.

Migration pattern and fate of neural crest cells

The neural crest is a transitory structure of the vertebrate embryo arising from the lateral ridges of the nervous primordium and giving rise, among other cell types, to the neurones and supporting cells of the peripheral nervous system (sensory ganglia of the head and the rachis and autonomic sympathetic and parasympathetic ganglia), to the melanocytes and to mesenchymal derivatives in the head. As the elements derived from the neural crest are dispersed in various locations, the neural crest cells have to migrate throughout the developing body before reaching the embryonic rudiment in which they differentiate. This structure is, in many ways, an attractive model for the study of certain developmental problems and the fate of its cells has been widely investigated. Although they yielded much important information, the experimental techniques used by earlier workers for investigating differentiating capabilities of crest cells (excision and electrocauterization of the neural anlage, and various cell labelling techniques: see refs 6, 10, 11) did not provide a complete and detailed picture of the migration pattern and the developmental fate of the whole population derived from the crest. The application of the quail-chick marker to this problem brought additional information on the role of the neural crest in the construction of the head in higher

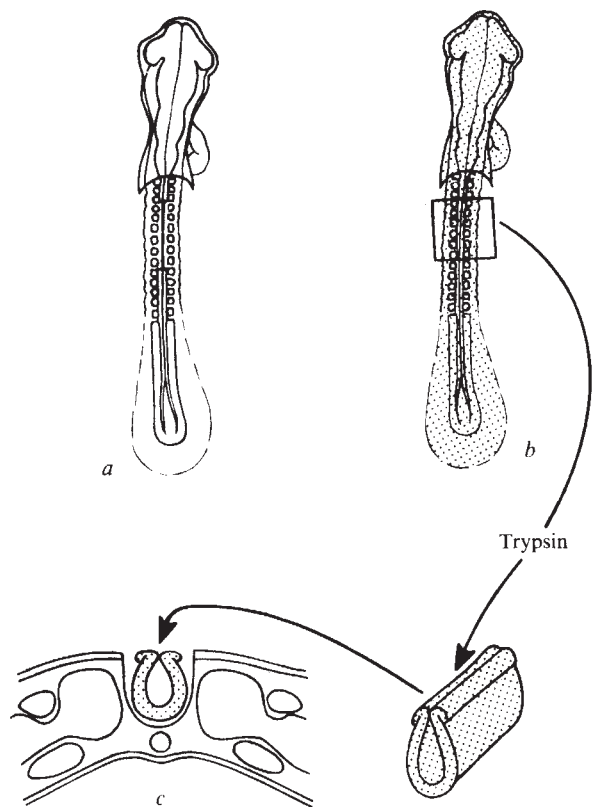


Fig. 1 Schematic representation of the isotopic-isochronic grafting technique of neural primordium between quail and chick embryos. *a*, Chick host embryo following the removal of the neural anlage from somite 4 to somite 10. *b*, Quail donor embryo from which the corresponding fragment of the neural tube + neural fold is isolated by enzymatic digestion. *c*, Graft of the quail neural primordium into the chick embryo.

vertebrates¹²⁻¹⁶ and allowed new crest derivatives to be identified¹⁷⁻¹⁹. The method used, derived from that described by Weston²⁰, involved (1) surgical removal of a defined region of the neural primordium in a host embryo before the onset of crest cell migration at this level; and (2) its replacement by the strictly equivalent neural area originating from a donor embryo of the alternative species, at the same developmental stage (that is, isotopic and isochronic grafts) (Fig. 1). Since the graft is correctly positioned in the host, it develops normally in the chimaera (Fig. 2). Furthermore, the regulation processes that often follow extirpation of embryonic territories do not take place in the chimaera since the empty space is occupied by an equivalent structure originating from the donor embryo. Immunological mechanisms do not function during embryonic life and tissues of the two species are thus tolerated by each other. In the cephalic region, where the neural fold is larger than in the trunk, selective substitution of pieces of the neural crest alone can be carried out^{12,13}. Migration of crest cells is followed on serial sections of the host embryos at various developmental stages. Recognition of the phenotype displayed by the grafted cells requires identification of both nuclear characteristics and an appropriate differentiation marker in the same cell. Electron microscopy can sometimes do this but, more often, the successive application of a cytochemical method (for example Falck's technique to evidence catecholamines²¹) and the Feulgen-Rossenbeck stain on the same section, have to be used.

Differentiation of the peripheral nervous system

Cells originating from the neural crest differentiate into constituents of two major neuronal systems: the sensory and the autonomic. Considerable interest has been focused in recent

years on the development of autonomic nerve cells and particularly on their chemical differentiation in terms of neurotransmitter synthesis. In complementary approaches the migratory behaviour and capabilities for differentiation of the presumptive autonomic neuroblasts have been studied by means of transplantation experiments *in vivo*, and the stability of neurotransmitter synthesis in developing autonomic neurones has been examined *in vitro*.

Although the ganglia of the autonomic nervous system and the adrenomedullary cells have long been recognized to be of neural crest origin, the migration pathways followed by their precursor cells were controversial (see ref. 22). By using the quail-chick chimaera system, we investigated their origin in normal development in the avian embryo. Isotopic and isochronic grafts of small fragments (corresponding to a length of 4-6 somites) of quail neural primordium into chick embryos (and vice versa) were systematically carried out along the whole length of the neural axis. The migrating neural crest cells were thereafter observed on serial sections of the host trunk (in the sympathetic chain and the adrenal medulla) and digestive tract (in the enteric and intravisceral ganglia). A correspondence could be established between the level of the graft and the definitive location of ganglion cells. In this way, the fate map of the autonomic cell precursors could be established (Fig. 3).

One of the striking observations made during these experiments was the finding that in the cervico-dorsal region (between somites 7 and 28) neural crest cell migration was strictly confined to the dorsal mesenchyme derived from the somites and the intermediate cell mass. Except for the melanocytes and for the Schwann cells that followed the nerve bundles to the periphery, neural crest derivatives were restricted to the sensory and sympathetic chain ganglia, the aortic and adrenal plexuses and the adrenomedullary paraganglia. No ganglion cell precursors penetrated the dorsal mesentery to contribute to either the ganglion of Remak or the enteric plexuses. In contrast, isotopic grafts carried out at the vagal (from somites 1 to 7) and lumbosacral (behind the 28th somite pair) levels of the neural primordium resulted in colonization of both the dorsal mesenchyme and the splanchnopleure. The myenteric plexuses are derived mainly from the crest at the level of somites 1-7; the contribution of the lumbosacral crest is restricted to the ganglion of Remak and to some ganglion cells of the post-umbilical gut. Crest cells continue to migrate ventrally from the vagal region between the 7- and 14-somite stages (approximately) and become incorporated into the mesodermal wall of the fore-gut, where they subsequently undergo a long cranio-caudal migration. Penetration of the hind-gut by either vagal or lumbosacral crest cells does not occur before days 7 to 8 of incubation^{22,23}.

The neural crest can therefore be divided into several different axial regions with respect to the development of the autonomic nervous system. One (level of somites 1-7) gives rise



Fig. 2 Transverse pigmented stripe resulting when a fragment of a quail neural tube was grafted into a White Leghorn chick embryo.

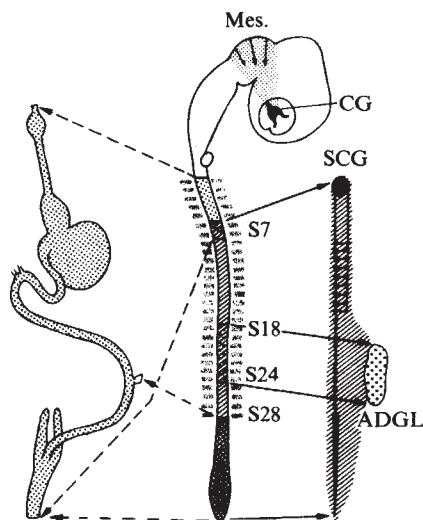


Fig. 3 Diagram showing the origin of adrenomedullary cells and autonomic ganglion cells. The spinal neural crest caudal to the level of the fifth somite gives rise to the ganglia of the orthosympathetic chain. The adrenomedullary cells originate from the spinal neural crest between the level of somites 18–24. The vagal neural crest (somites 1–7) gives rise to the parasympathetic enteric ganglia of the pre-umbilical region, the ganglia of the post-umbilical gut originating from both the vagal and lumbosacral neural crest. The ganglion of Remak is derived from the lumbosacral neural crest (posterior to the 28th somite level). Ciliary ganglion (CG) is derived from the mesencephalic neural crest. ADGL, adrenal gland; Mes., mesencephalon; SCG, superior cervical ganglion.

to the enteric ganglia of the gut. Another (somites 7–28) is the precursor of sympathoblasts of the trunk (within this area, the 'adrenomedullary' level of the crest contributes to the adrenal glands) and, finally, there are two regions from which both enteric and sympathetic ganglion cells arise (somites 5–7 and behind the 28th pair of somites). Next, neural primordia were transplanted heterotopically between quail and chick embryos, as shown in Fig. 4. This was to find out whether the migratory behaviour and phenotypic expression of crest cells from these regions were irreversibly fixed before migration and if experimental rearrangement of the axial levels of the neural crest would lead to developmental disturbances in the distribution and development of crest cell derivatives. Analysis of the resulting chimaeras showed that the neural crest cells of the adrenomedullary region, grafted at the vagal level, were able to colonize the gut (which they never do in normal development) and give rise to functional cholinergic enteric ganglia; second, neural crest cells from the quail mesencephalon or rhombencephalon, grafted at the chick adrenomedullary level, produced adrenergic sympathetic ganglia, populated the adrenal gland and differentiated into adrenomedullary-like cells^{24,25}. This indicated that the migratory behaviour of the crest cells depends on the pathways available when they leave the neural primordium rather than on some specificity related to their origin in the neural axis. The vagal and the adrenomedullary regions of the embryo provide preferential pathways leading crest cells to the gut and to the suprarenal gland, respectively. In addition, the phenotypic expression of the crest cell population is regulated by environmental factors which elicit either cholinergic or adrenergic cell differentiation, irrespective of their fate in normal development. Furthermore, the potentialities for giving rise to cholinergic parasympathetic cells, adrenergic sympathetic ganglia and adrenomedullary paraganglia are not restricted to the areas identified in the isotopic-isochronic experiments, but are present in the entire crest.

Two questions thus arise. First, do the environmental signals for differentiation act on neural crest cells during their migration or after they have reached their definitive location? And second, when do the developing autonomic neurones become irreversibly differentiated into adrenergic or cholinergic cells?

When is the chemical differentiation of the autonomic neurone precursor determined?

To determine if chemical differentiation occurs before migration, we investigated the kind of transmitter synthesized by neurones when their extraintestinal migratory phase was eliminated. Since neural crest cells reach the hind gut only at 7 to 8 days of incubation, the colorectum remains totally aneural if it is removed from the embryo before this stage and grown on the CAM. When such an aneural rectum was associated with trunk neural crest for 10 to 12 days on the CAM, the crest cells migrated into it and differentiated into Auerbach's and Meissner's plexuses, in which catecholamine (CA)-containing cells were never observed. In contrast, significant levels of choline acetyltransferase (CAT) and acetylcholinesterase (AChE) activities were present in the explants^{7,26}. Thus the induction of cholinergic metabolism does not depend on environmental signals received by autonomic neurone precursor cells during migration.

In contrast, the expression of the adrenergic phenotype in sympathoblasts does seem to be influenced by developmental cues during the dorso-ventral migration of the neural crest cells. By associating the neural crest with various trunk structures, Cohen²⁷ and Norr²⁸ reached the conclusion that the crest cells receive differentiating signals on their route and that the notochord, ventral neural tube and somitic mesenchyme are of decisive importance in promoting CA-synthesis in crest neuroblasts.

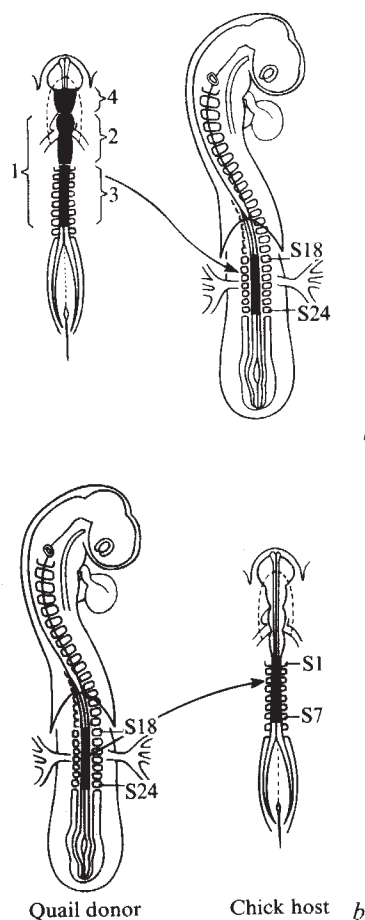


Fig. 4 Heterotopic grafts of quail neural primordium into the chick embryo. *a*, The quail primordium is removed from the mesencephalon; the anterior rhombencephalon or at the vagal level and grafted into the adrenomedullary region. *b*, The neural tissue is taken at the adrenomedullary level and grafted at the vagal level.

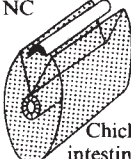
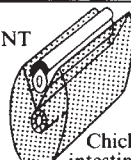
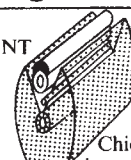
Quail NC	Enteric ganglia	CAT activity	Adrenergic ganglia
1  Chick intestine	+	+	-
2  Chick intestine	+	Not determined /	-
3  Chick intestine Chick notochord	+	Not determined /	+

Fig. 5 Association of the aneural colorectum of a 5-day chick embryo with various dorsal trunk structures of 2-day embryos: (1) chick intestine + quail neural crest (NC). (2) chick intestine + quail neural crest and neural tube (NT). (3) chick intestine + quail neural crest/neural tube and chick notochord. CAT, choline acetyltransferase. Cholinergic ganglia develop in all cases. Adrenergic ganglia differentiate only in explants containing the notochord.

It is striking that the first CA-containing neuroblasts appear in the somitic mesenchyme (in fact in the sclerotomal part of the somite) in the immediate vicinity of the notochord and of the dorsal aorta. We therefore decided to reinvestigate the respective roles of each of these dorsal trunk structures (neural tube, notochord and somitic mesenchyme) in sympathoblast differentiation. When trunk neural primordium was isolated and inserted in aneural gut wall mesenchyme (Fig. 5), the neural crest gave rise to enteric plexuses; but in spite of the presence of the neural tube in the explants, no CA-containing cells could be detected in the ganglia. The result was therefore similar to the differentiation observed when the neural crest alone was associated with the gut²⁶. In contrast, the inclusion of the notochord in the tissue associations resulted in the development of cholinergic myenteric plexuses and also in the formation of a series of adrenergic ganglia, located in the wall of the gut and characterized by the greenish fluorescence of CA. It is therefore clear that among the dorsal trunk structures, the notochord is of decisive importance in eliciting adrenergic differentiation in sympathoblasts, since, in its presence, adrenergic cells develop in the splanchnopleural gut mesenchyme, which normally supports only cholinergic differentiation. Moreover, this finding is in contradiction with the conclusions previously reached by Cohen²⁷ that the somite was the only structure of the embryo able to provide a convenient mesenchymal environment for adrenergic differentiation of crest-derived neuroblasts. In fact, culture experiments (unpublished) show that both the notochord and the sclerotomal part of the somitic mesenchyme are equally able to induce CA metabolism in the crest neuroblasts. This accounts perfectly for the distribution of the adrenergic cells in the trunk structures of the early embryo.

A recent observation made by Cocharde *et al.*²⁹ in the rat embryo supports the notion that notochord induces the adrenergic phenotype. Both tyrosine hydroxylase and CA first appear at 11.5 days of gestation in the sympathetic ganglia of the trunk. But, in addition, cells with an adrenergic phenotype are transiently detectable in the gut wall from day 11.5 to about day 14.5. Thus CA differentiation in these cells may have been induced by interactions with the somite-notochord-neural tube environment during their dorso-ventral migration towards the gut. The resulting adrenergic phenotype is expressed for a time after cells have left the dorsal trunk structures and while they are

pursuing their migration in the gut, but it cannot be maintained in the gut, either because of the lack of appropriate stimulation, or because other factors intervene.

Lability of transmitter phenotype in developing autonomic neurones

If the neurotransmitter phenotype of neural crest cells is selected by the tissue environment during early development what is the stability of the chemical differentiation in the developing autonomic neurones? We have investigated this by attempting to change the fate of already differentiating ganglion cells.

Cholinergic ciliary ganglion from 4.5–6 day quail embryos were incubated and implanted into the dorsal trunk environment of a 2-day chick host at the level of somites 18 to 24, the idea being to subject the maturing cholinergic cells to the adrenergic signals normally operating from day 3 to 4 on the precursors of sympathoblasts and adrenomedullary cells (Fig. 6). The ciliary ganglion, which normally never contains adrenergic cells at any stage, exhibited cholinergic activity at the time of grafting^{30–33}. Examination of the host embryos at 8 to 10 days showed that, irrespective of its age when grafted, the ciliary ganglion never remained intact at the site of implantation. Quail cells were found dispersed in the host trunk structures, but not randomly: they were always localized exclusively in normal sites of neural crest cell arrest and participated in the formation of various host crest derivatives, that is sympathetic ganglia, aortic and adrenal plexuses, adrenomedullary cords, ganglion of Remak and enteric plexuses. However, quail cells were virtually never found in the host sensory ganglia; in contrast, the Schwann cells of the rachidian nerve were mostly of the quail type at the site of the graft.

The ability of cells of a differentiating ganglion to migrate anew when grafted into the microenvironment which normally sustains crest cell migration is remarkable enough. However, the result obtained by treating the sections with the combined FIF–Feulgen–Rossenbeck techniques was even more striking: the quail cells which had homed to the sympathetic ganglia, the aortic and adrenal plexuses and the suprarenal gland exhibited the bright greenish fluorescence of CA (Fig. 7). In electron microscopy, most of the quail cells of the suprarenal gland, recognizable by their large DNA-rich nucleolus, were also seen to contain the electron-dense secretory granules characteristic of adrenomedullary cells.

Although in some cases sympathetic chain ganglia in the host were found to be totally (or mainly) made up of quail cells, grafted and host cells were usually intermingled. The possibility that the fluorescence observed in the quail cells resulted merely from an uptake of CA released by host sympathoblasts was ruled out by experiments in which the host neural crest was removed before the graft of the quail parasympathetic ganglion. In this case, adrenergic nervous structures entirely made up of quail cells developed at the site of the operation and showed the same CA fluorescence as in the previous series³³.

The cells of the ciliary ganglion which colonized the splanchnopleure and became localized in the host Remak and enteric ganglia were not CA-positive after FIF treatment, and presumably developed as cholinergic cells.

In recent (unpublished) experiments, pieces of ciliary ganglia taken from quail embryos as old as 9 days of incubation were similarly grafted into 2-day chick embryos. Quail cells were found in the same locations in the host structures as in the previously described experiments except that their migration was practically restricted to the dorsal trunk and generally did not penetrate the dorsal mesentery. Many quail cells were found with CA fluorescence in the sympathetic host ganglia in the adrenal gland and in the aortic plexuses.

We are currently examining in detail the ability of the grafted cells to proliferate in a young host. Merely from the extent of quail cell distribution in the 8-day host following the graft of a 4.5- to 6-day-old ciliary ganglion, it is clear that their number is higher than the total number of neurones of the mature ciliary

ganglion. Detailed counts of the number of quail cells labelled one hour after ^3H -thymidine injection at various intervals after grafting, show that many mitoses occur both during the migration of ciliary ganglion cells and after their stabilization in host structures. Numerous quail neurones and adrenomedullary cells are labelled with ^3H -thymidine at all stages up to 9 days of incubation of the host. These observations are in agreement with the recent findings³⁴ that developing sympathoblasts, actually synthesizing CA, divide until late in development.

Such proliferative potential is not maintained as long in the cholinergic ciliary ganglion since, according to the work of Landmesser and Pilar³¹, mitotic activity has virtually stopped in chick neuroblasts at stage 25 (4.5 to 5 days of incubation), while the definitive number of neurones, after physiological cell death has occurred, is reached around day 12. Therefore, transplantation of a cholinergic ganglion into a younger host changes its developmental program profoundly enough to modify not only its chemical differentiation but also the number of cell cycles that the neuroblasts undergo before reaching maturity.

In summary, our results demonstrate that CA synthetic ability exists in cells of developing cholinergic ganglia; although not expressed in normal development, it can be elicited by the dorsal trunk structures and the adrenal gland environment.

Since all our experiments deal with the transplantation, not of single cells but of a population, our results do not allow us to state that the appearance of CA-producing cells results from the conversion of initially cholinergic cells. It may well be that the ciliary ganglion, even as late as 9 days of incubation, contains a pool of still undifferentiated neuroblasts which may be destined to disappear later on in normal development. It should nevertheless be emphasized that the capacity for a developing

autonomic neurone to change its transmitter metabolism in response to environmental influences has been indisputably demonstrated by experiments *in vitro*³⁵⁻³⁸.

Manipulation of the fluid environment in which dissociated post-mitotic neurones from the newborn rat superior cervical ganglion are growing has been shown to influence the choice of transmitters and the type of synapse they make. These elegant experiments (reviewed by Patterson³⁹) have revealed the existence of a soluble factor mediating the conversion of an adrenergic to a cholinergic phenotype. Moreover, during the switch in transmitter metabolism in culture, neurones with both adrenergic and cholinergic functions have been identified in single-cell cultures^{40,41}.

Although cell physiology may be profoundly altered by culture conditions, the results obtained *in vitro* are important in defining the mechanisms regulating autonomic nervous system development *in vivo*, particularly in two respects. First, the discovery that a change in transmitter synthesis can occur in post-mitotic nerve cells and the demonstration of dual-function neurones in culture strengthen the view that in the transplantation experiments *in vivo*, the environment imposes the choice of phenotypic expression on bipotential neuroblasts. The alternative hypothesis, that the microenvironment merely selects from a mixed population of committed adrenergic and cholinergic neuroblasts, becomes more unlikely. If the dual-function neurones detected in culture represent a transitional state between the adrenergic and the cholinergic condition, it seems probable that a similar process takes place in reverse when the cholinergic ciliary ganglion cells become adrenergic after back-transplantation into a younger host. Second, the demonstration that cholinergic differentiation can be triggered *in vitro* by a factor of non-neuronal cell origin suggests that an analogous mechanism might operate *in vivo*. In particular our experiments suggest that adrenergic differentiation is regulated by 'factors' produced by cells of the notochord or by the sclerotomal part of the somite and released into the extracellular matrix. Alternatively, an integral membrane component of the somitic mesenchyme might influence crest cell differentiation through intimate cell-cell contacts. The developmental relationships between sclerotomal and neural crest cells during ontogenesis support such a hypothesis but, experiments involving cultures of neural crest *in vitro*, to be described below, show that such cell-cell contacts are not necessary for eliciting CA metabolism in the developing sympathoblasts.

Developmental capabilities of neural crest cells *in vitro*

From their study of the *in vitro* differentiation of crest cells in the absence of any other tissue of the embryo, Greenberg and Schrier⁴² reported CAT activity in mesencephalic neural crest cells after 7 days in culture and Cohen⁴³ described the appearance of adrenergic cells in trunk crest cultures. In both cases, the total neural anlage was explanted for 48 hours and, after the migration of crest cells had taken place, the neural tube was mechanically removed. This technique cannot avoid possible contamination of the crest cultures by central neurones or possible influence of the neural tube itself on peripheral neuroblast differentiation⁴⁴. Therefore our approach has been to excise neural crest from quail embryos, at both mesencephalic and truncal levels, by a microsurgical procedure that leaves the neural tube in place. Cultures of pure crest explants from each region underwent morphological differentiation and were able to synthesize both ACh and CA. Although the amount of neurotransmitter synthesized could be increased by co-culturing with a variety of embryonic tissues, the ratio of ACh to CA formed in a given culture was markedly dependent on the composition of the liquid medium. In particular, horse serum was found to favour ACh synthesis, whilst fetal calf serum preferentially enhanced CA production. These studies, together with those of other workers^{42,43}, strongly suggest that direct intercellular contact with non-neural tissue is not essential for inducing neurotransmitter synthesis.

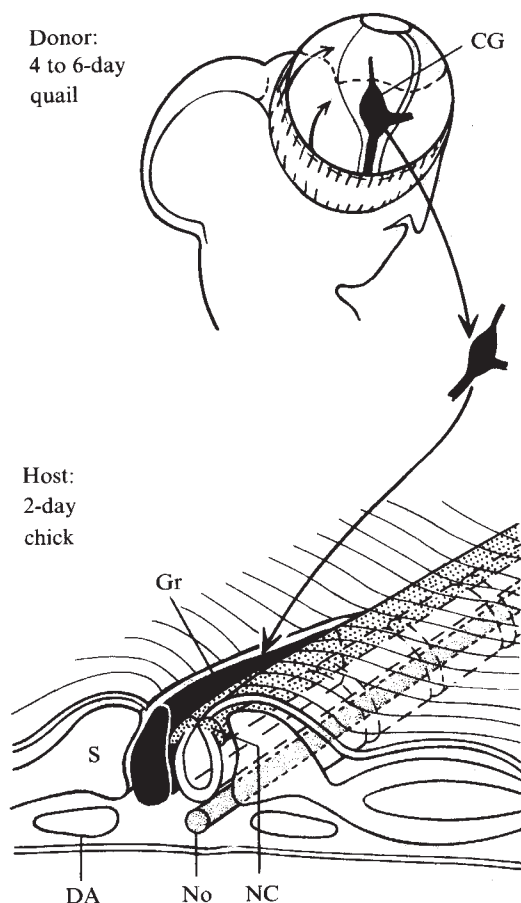


Fig. 6 Experimental design for back-transplantation of differentiating cholinergic ganglia from 4- to 6-day quail donor embryos into 2-day chick hosts. The ciliary ganglion (CG) is dissected and inserted either whole or in pieces into a slit made between the neural primordium and the somites (S). DA, dorsal aorta; Gr, graft; NC, neural crest; No, notochord.

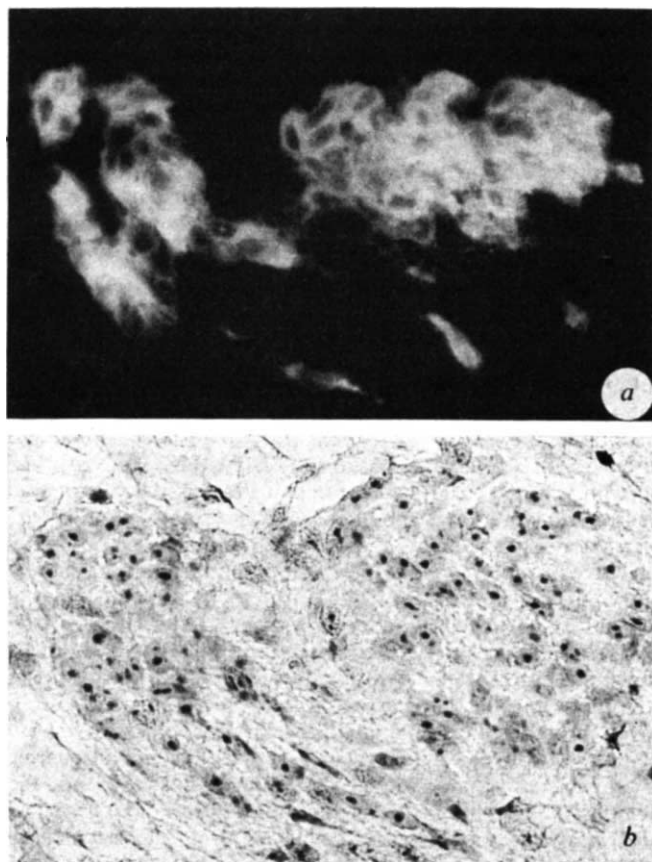


Fig. 7 Result of the experiment of back-transplantation of a quail autonomic ganglion into a 2-day chick embryo (as represented in Fig. 6). Transverse section of the host at 7 days of incubation at the level of the sympathetic ganglia. *a*, CA-containing cells shown after application of Falck's technique. *b*, Post-staining of the same section with the Feulgen reaction shows that the fluorescent cells originate from the grafted ciliary ganglion since they have the nuclear marker. $\times 460$.

The choice between the cholinergic and the adrenergic pathways made *in vivo* by future neuroblasts could thus be the result of selective induction by soluble factors encountered at the opportune moment. If such an interpretation is correct, one has to assume that differentiating factors for both ACh and CA synthesis are present in the culture medium. They might be provided by the serum and/or by the embryo extract. Another possibility is that neuroblast precursors have the ability to synthesize neurotransmitters while still in the neural primordium; the role of environmental cues would then be limited to stabilizing and enhancing the appropriate phenotype.

As far as the adrenergic system is concerned, a number of histochemical studies have shown that the earliest detectable appearance of tyrosine hydroxylase and of CA synthesis in presumptive sympathoblasts coincides with their aggregation into the primary sympathetic chain^{29,45,46}. This suggests that the first of the above mentioned alternatives is correct and that an inductive event occurs during the crest cells' dorso-ventral migration which gives them a bias towards the adrenergic pathway. On the other hand, recent results from our laboratory cast serious doubt on the applicability of this hypothesis to cholinergic differentiation⁴⁷. We were, in fact, able to show that mesencephalic crest, excised from quail embryos of 8–12 somites (when the crest appears as an actively migrating sheet of cells under the ectoderm⁴⁴) is able to convert ³H-choline to ³H-ACh and that this transformation is due to CAT. Furthermore, this activity can also be detected at an even earlier stage (5–7 somites), when migration is just beginning. In other words, mesencephalic crest cells already contain a cholinergic system

when they start to migrate. We cannot yet say whether all or only some of the crest cells are involved. However, if we assume that the early cholinergic differentiation concerns the presumptive parasympathetic neuroblast (the ciliary ganglion is derived from the mesencephalic crest^{13,48}), the obvious implication is that no inductive stimulus is necessary once the latter have reached their definitive site, and cholinergic maturation involves consolidation and augmentation of already acquired properties. There is one clear case in which external cues have been shown to stabilize synthesis of a particular kind of transmitter and decrease the lability of neuronal chemical differentiation: Walicke *et al.*⁴⁹ have shown that the chemical differentiation of sympathetic neurones is stabilized by neuronal activity itself.

Conclusions

In our laboratory and elsewhere quail-chick chimaeras have proved most useful in the investigation of the early stages of the differentiation of the nervous system^{48,50,51}. We have already identified some new derivatives of the neural crest and analysed certain aspects of the development of the autonomic nervous system; comparison of the *in vivo* transplantation and *in vitro* culture experiments has been particularly fruitful.

Future experiments will explore the developmental relationships which may exist between the neuroblasts that give rise to the sensory neurones of the dorsal root ganglia and those which become autonomic cells. It would be of great interest to know when the commitment of these different cell types occurs and whether the choice in this case also, remains labile for a while. The relationships between the glial satellite cells of the ganglia and the neural cell lines will also be examined. Furthermore the chemical differentiation of the peripheral neurones needs to be reconsidered in terms of neurotransmitters other than just ACh and CA and including neuropeptides.

Note that the chimaeric cell labelling technique has also been of great value in analysing many other aspects of embryonic development such as the ontogeny of the haemopoietic system^{52–59}, the development of the kidney^{60,61} and limb^{62–67} and the early movements of cells in gastrulation^{68,69}.

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ARTICLES

An immunoglobulin deletion mutant with implications for the heavy-chain switch and RNA splicing

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The IF2 immunoglobulin mutant is a DNA deletion of one coding segment and large sections of the flanking intervening sequences. The deletion results in a new splicing pattern and starts in a DNA region containing tandemly repeated sequences which may carry heavy-chain class switch signals.

IF2 WAS the first structurally defined deletion mutant of eukaryotic cells growing in tissue culture¹. It was isolated as a spontaneous mutant secreting an immunoglobulin of altered isoelectric point². Its phenotype was defined by sequence analysis¹, mRNA size³ and mRNA sequence⁴ as due to an internal deletion of the $\gamma 1$ heavy chain of the parental myeloma protein secreted by the cell line P3 (a line of the tumour MOPC 21 (ref. 5) adapted to tissue culture⁶). Internal deletions of immunoglobulin heavy chains are characteristic of a myeloma-like disease, the human heavy-chain disease (HCD)^{7,8}, and IF2 represents an analogous example in the mouse. We show here that the genetic origin of the protein deletion is a large deletion at the DNA level. The deletion involves at least 5.5 kilobases, including an entire segment of coding sequence (exon) and parts of the intervening sequences (introns). The consequence is a new phenotype resulting from the splicing of two exons not normally ligated together, demonstrating the importance of colinearity of splicing points.

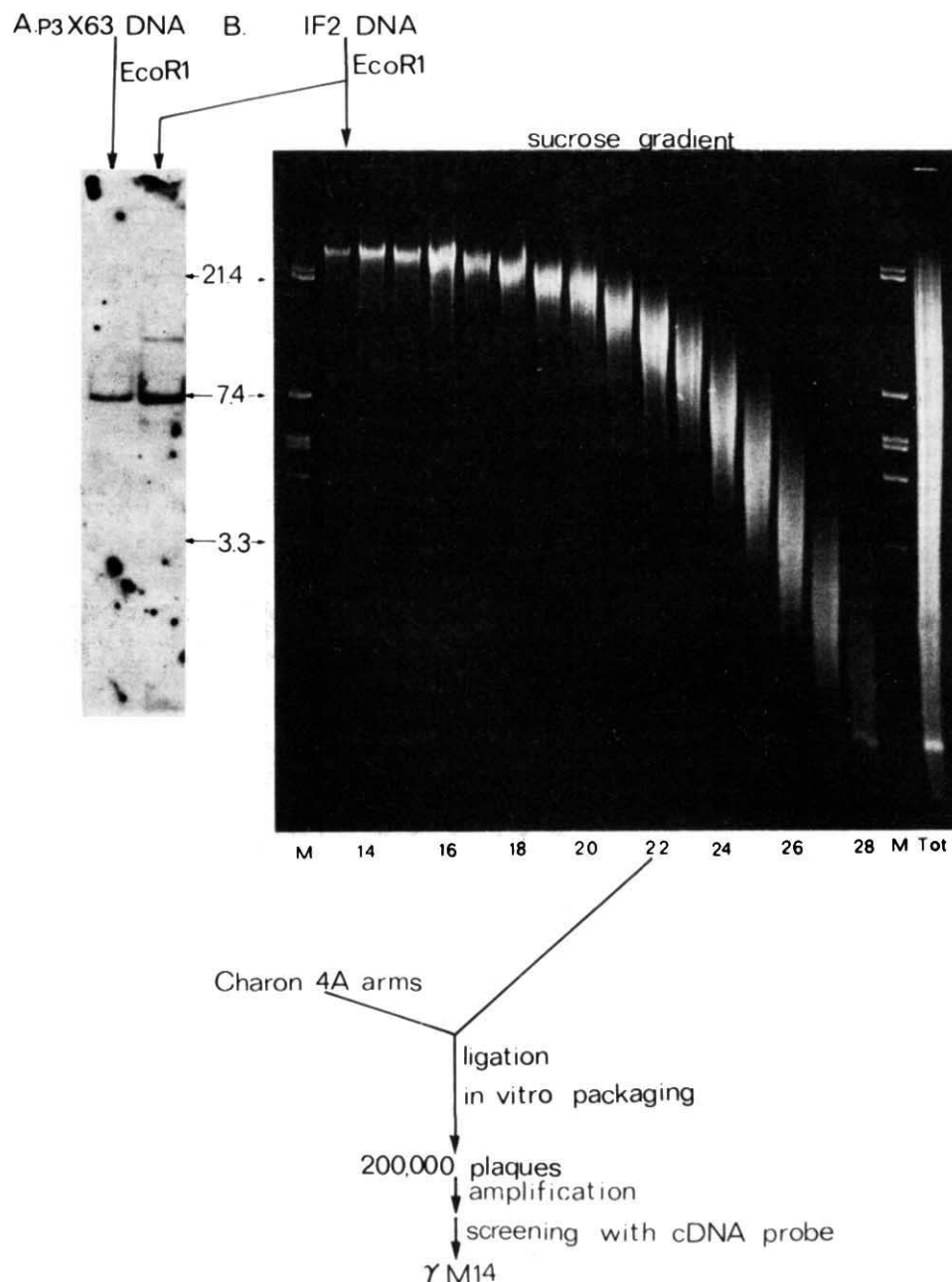
The segments of DNA at which the deletion begins and ends have been studied in detail. The present study leads us to conclude that those points are relevant to an important event

taking place during maturation of the immune response, the heavy-chain switch. For this reason, experiments are also described to explore further the correlation between the IF2 deletion and the class switch.

The IF2 mutation is a DNA deletion in the heavy-chain gene

The constant (C) region of the $\gamma 1$ heavy chain (the subclass secreted by P3 and its mutant IF2) is divided into three domains of primary and tertiary structure homology, designated CH1, CH2 and CH3 from the amino- to the carboxy-terminal end. Between the CH1 and the CH2 domains there is a segment, the hinge region, of about 13 amino acids, which includes all the inter-chain disulphide bridges. The IF2 protein deletion encompasses exactly the CH1 domain. The gene for the $\gamma 1$ region contains four segments of DNA coding for each of the domains and the hinge (exons) separated by intervening sequences (introns)^{9,10}. As the exons are spliced together, the CH1 protein deletion could be the result of either a DNA deletion of part or all of the CH1 exon, or the result of another

Fig. 1 Isolation of a recombinant DNA clone containing $\gamma 1$ coding sequences from IF2 cells. *a*, Identification of *EcoRI* fragments containing $\gamma 1$ sequences. High molecular weight DNA from P3-X63 cells (left) or IF2 cells (right) was digested with *EcoRI*, using as an internal control the digestion pattern of phage λ DNA. The digested mouse DNAs were fractionated on a 0.8% agarose gel and transferred by blotting onto nitrocellulose filters⁴⁰. The filters were pretreated and hybridized in $1 \times \text{SSC}$, 0.9% dextran sulphate to nick-translated $\gamma 1/\text{A5}$ (ref. 4) DNA as previously described^{11,41,42}. The filters were washed¹¹ and autoradiographed for 14 days at -70°C , using preflashed film and tungstate intensifying screens⁴³. *b*, Partial purification and cloning of the IF2 mutant gene. High molecular weight DNA (600 μg) from IF2 cells was digested with *EcoRI*, extracted with phenol and precipitated with ethanol. The sample, dissolved in 0.25 ml 50 mM NaCl, 20 mM EDTA, was fractionated on a 10–30% sucrose gradient containing 1 M NaCl, 1 mM EDTA, 10 mM Tris (pH 7.5) in the SW27 rotor (25,000 r.p.m., 23 h, 20°C). The gradient was fractionated into 39 fractions, and aliquots of fractions 13–28 were analysed on a 0.8% agarose gel, using *EcoRI*-digested λ DNA (M) as size markers. Also present on the gel was *EcoRI*-digested IF2 DNA which had not been fractionated on a sucrose gradient (Tot). The DNA was visualized by ethidium bromide staining. Charon 4A DNA¹² was digested with *EcoRI* and the left- and right-hand fragments (19.8 and 10.8 kilobases) were purified on sucrose gradients as described above. About 4 μg of these Charon 4A 'arms' were ligated to 4 μg of fraction 22 of the *EcoRI*-digested, fractionated IF2 DNA (above), and the resulting DNA was packaged into phage particles *in vitro*^{44–46}. The packaged DNA was used to transfect *E. coli* strain 803 sulI⁺suII⁺; about 200,000 plaques were generated. The top agar containing these plaques was scraped into culture media, and a phage stock was prepared⁴⁴. After this amplification process, about 400,000 plaques were screened by the Benton–Davis procedure⁴⁷. Hybridization to nick-translated $\gamma 1/\text{A5}$ was in $6 \times \text{SSC}$ (no dextran sulphate). Positively hybridizing plaques were reperfired twice as individual plaques. Three resulting plaques which hybridized to $\gamma 1/\text{A5}$ were designated γM1 , γM5 and γM14 .



event leading to the incorrect splicing of the V-region exon to the hinge exon. To investigate these two alternatives, we have studied the structure of the IF2 DNA.

The $\gamma 1$ genes of IF2 cells and of the clone P3-X63 from the parental cell line were compared by the Southern filter hybridization technique, using as a probe the cloned IF2 heavy-chain cDNA⁴. Two fragments hybridize to the cDNA probe in *EcoRI*-digested P3-X63 DNA: a 6.8-kilobase fragment which contains the $\gamma 1$ wild-type constant-region coding sequences^{9,10}, and a 20-kilobase fragment which has been assigned to the $\gamma 2a$ coding sequences¹¹ (Fig. 1a). In addition to these fragments, the cDNA clone hybridizes to more fragments in *EcoRI*-digested IF2 DNA, a 15-kilobase fragment and weakly to a 6-kilobase fragment (which we designate the 6.0 RI fragment). We have partially purified the 15-kilobase fragment by sucrose gradient fractionation after *EcoRI* digestion (Fig. 1b). The enriched fraction was ligated into the λ vector Charon 4A (ref. 12) and packaged *in vitro*, and about 200,000 plaques were amplified

and 400,000 plaques screened. Three clones which hybridized to the cDNA clone were purified and grown in bulk for analysis. The three clones seemed to be similar, in that their restriction patterns after *EcoRI* digestion were identical (not shown). One (γM14) was analysed extensively.

A restriction map of γM14 was constructed using single and double digests with *EcoRI*, *Bam*HI, *Hind*III, *Xba*I and *Kpn*I (Fig. 2a). The IF2 DNA insert includes four *EcoRI* fragments. Therefore, the γM14 clone seems to be a product of partial *EcoRI* digestion, in spite of the fact that the IF2 DNA was apparently digested to completion, as shown by the digestion of an internal control phage λ DNA. We have isolated an independent, but identical clone by a second *EcoRI* digestion of IF2 DNA and screening of Charon 4A clones prepared from unfractionated IF2 DNA. Thus, it is very unlikely that the four *EcoRI* fragments came together by random re-ligation.

Southern hybridization analysis showed that the IF2 heavy-chain cDNA hybridized to the 6.0 RI fragment and to two *Pst*I

fragments of about 1.8 and 1.6 kilobases from γ M14 DNA. More extensive restriction enzyme and Southern hybridization analysis (which will be discussed below) located the two *Pst*I fragments within the 6.0 RI fragment (Fig. 2b). These two *Pst*I fragments were isolated and partially sequenced by a combination of the dideoxy method on phage M13 subclones^{13,14} and the Maxam-Gilbert method on a pBR322 subclone¹⁵ (see Fig. 2b legend). These sequences demonstrated that the two *Pst*I fragments contained the γ 1 hinge, CH2 and CH3 coding sequences and appropriate introns arranged as shown in Fig. 2b. Part of these sequences, including the hinge exon and about 80 nucleotides to the left of the hinge exon (that is, in the intervening sequence towards the V region), are shown in Fig. 2c. The sequence derived from the γ M14 clone agrees with that of the γ 1 gene^{9,10} throughout the hinge exon and for the first 46 nucleotides to the left of the hinge coding sequence (with a single A for G discrepancy). Forty-six nucleotides into the intervening sequence, the two sequences diverge completely.

The nucleotide sequences presented in Fig. 2c and others we have completed (Fig. 2b) demonstrate that the γ M14 recombinant clone contains γ 1 coding sequences. They also indicate that the γ 1 gene included in γ M14, when compared with the parental γ 1 gene, has been altered in the intervening sequence between the CH1 and hinge exons. To determine the extent of this change, we have constructed a detailed restriction map of the IF2 γ 1 gene and compared this with the published restriction sites in the normal γ 1 gene^{9,10}. As expected from the sequences described above, the restriction sites (compare Fig. 3a and b)

lying to the right of the hinge correspond to those previously mapped in the wild-type gene^{9,10}. On the other hand, those to the left do not correspond at all to the sites to the left of the hinge in the γ 1 gene. In particular, the enzyme sites for *Bam*HI, *Xba*I, *Sac*I and *Hinc*II, which cut in the γ 1 CH1 exon, or just to its left, are missing or altered. These differences correlate with the loss of CH1 from the IF2 heavy chain¹. The restriction mapping data indicate that a deletion removes the whole of the CH1 exon, at least 3 kilobases of the intron to its left, and 0.3 (85%) of the intron to its right.

The V region of P3 cells and IF2 cells are identical¹. Therefore, the IF2 deletion must begin in the V-CH1 intervening sequence (after the V-region exon). The deletion results in the direct splicing of the V exon to the hinge exon. Insofar as can be estimated by gross yields of mRNA³, the IF2 cell splices the V exon to the hinge exon as efficiently as P3 cells splice V to CH1 and CH1 to the hinge. Thus, only the linear order of exons seems to determine which exons are joined together, as has been suggested by the sequences of junction regions. These results argue against a multiplicity of splicing enzymes, for the splicing machinery seems to be able to join any exon-intron boundary to any intron-exon boundary. As the same V region can be spliced with different C regions (the class switch), the argument of a single enzyme specificity applies to all heavy chains.

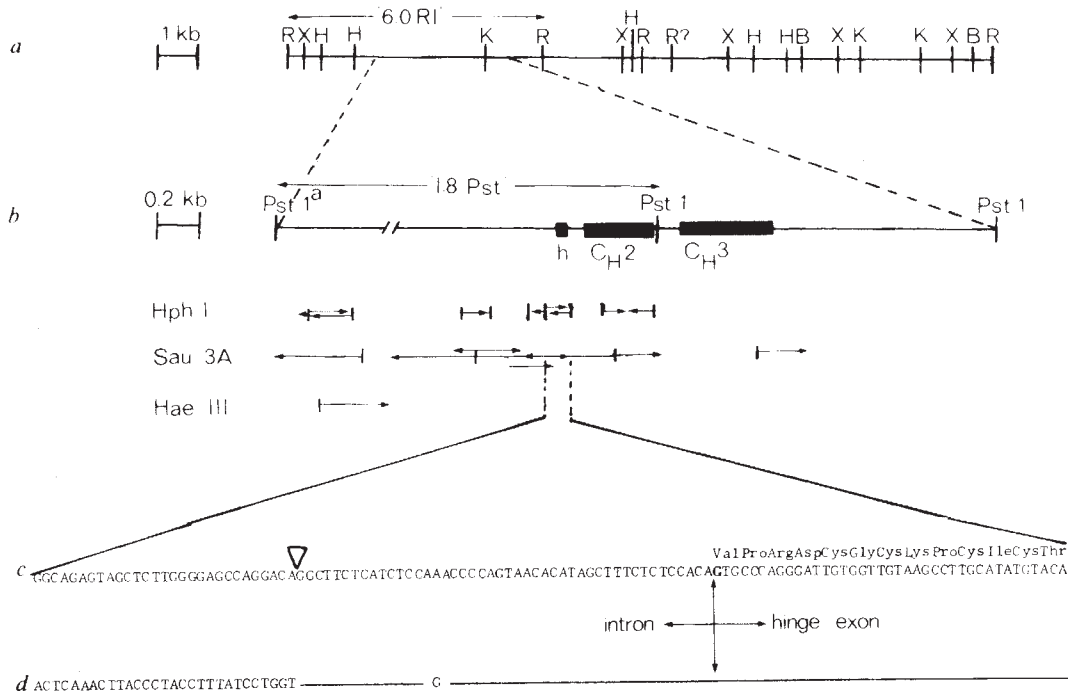
The fact that 85% of the CH1-hinge intron and at least 5 kilobases (see below) of the V-CH1 intron have been deleted without affecting splicing capability provides evidence against the importance of internal intron sequences in splicing. This also

Fig. 2 Restriction analysis

and sequencing of the recombinant clone γ M14. **a**, Restriction map of γ M14.

This map was constructed from the restriction pattern of single and double digests of γ M14 DNA, and single digests of *Eco*RI fragments eluted from agarose gels. The 0.7-kilobase *Eco*RI fragment could not be unambiguously placed, although partial *Eco*RI digestion of γ M14 DNA suggested its position to be between the 3.0- and 6.8-kilobase *Eco*RI fragments. The 6.0-kilobase *Eco*RI fragment (hereafter referred to as '6.0 RI fragment') hybridized to the γ 1/A5 probe. The γ M14 clone is a product of partial *Eco*RI digestion of IF2 DNA; a small amount of the 6.0 RI fragment (total digestion product) can be seen in Southern hybridization filters (Fig. 1a). **b**, Sequencing strategy. Two *Pst*I fragments from γ M14 (about 1.8 and 1.6 kilobases) hybridized to γ 1/A5 in a Southern filter hybridization experiment (not shown).

These fragments were prepared by electrophoresis in a 6% acrylamide gel (400×200×0.4 mm, 7 mA, 28 h) and eluted by soaking the gel slices in 0.3 ml 0.2 M sodium acetate, 0.5% SDS and 0.1 mM EDTA three times for 24 h each. The DNA was precipitated with the addition of 20 μ g yeast tRNA and ethanol. The 1.8-kilobase fragment, hereafter referred to as the '1.8 *Pst* fragment' was subcloned in the *Pst*I site of pBR322 (ref. 48); propagation was carried out in the disabled *E. coli* strain MRC-1 (GMA issue number 2-5). Some *Hph*I fragments were prepared from this plasmid (designated γ 1/IF2P.1.8) and sequenced by the method of Maxam and Gilbert¹⁵. Strand separation was accomplished by hybridization to appropriate single-stranded M13 DNA (see below and ref. 49). The 1.8- and 1.6-kilobase *Pst*I fragments were cut with *Sau*3A, and the subfragments were ligated into the *Bam*HI site of a derivative of M13mp2 containing a single *Bam*HI site⁵⁰ and an amber mutation (G. Winter, unpublished). The ligated DNAs were used to transform *E. coli* JM101 (ref. 50) and DNA samples from white plaques were sequenced using the dideoxy method^{13,14}. The fragments sequenced are shown in the lower part of Fig. 2a. Each arrow represents sequences obtained from an independent clone. A 1,200-base pair *Hae*III fragment from γ 1/IF2P.1.8 was purified on a 6% acrylamide gel, and about 0.5 μ g of this fragment was cloned in the *Eco*RI site of M13mp2 using synthetic *Eco*RI linkers¹⁴. DNA from a white plaque was sequenced by the dideoxy method^{13,14}. Note the break in the 1.8 *Pst* fragment, which we estimate to be 400 base pairs. The sequence derived is shown fully in Fig. 4. **c**, Sequences near the hinge coding region. The sequence of the hinge coding region (with amino acid sequence) and of the adjacent intervening sequence is shown. The intron between the hinge and CH2, the CH2 exon and part of the 3'-untranslated region of γ M14 have also been sequenced and correspond to the published γ 1 sequences from newborn mouse DNA¹⁰ and from the MOPC 21 myeloma DNA⁹. The following differences were noted (using the numbering system of Honjo *et al.*¹⁰): residues 800-801, we find three Ts rather than two¹⁰; residues 804-805, we find three Cs rather than two¹⁰; residues 809-811, we find four Cs rather than three¹⁰; residue 828, we find C rather than A¹⁰; residues 843-844, we find three Cs rather than two¹⁰; residue 1,754, we find T rather than C¹⁰; residue 1,821, we find G rather than C¹⁰. **d**, The sequence of the γ 1 germ-line gene⁸ near the hinge coding region. The line indicates concordance with the γ M14 sequence.



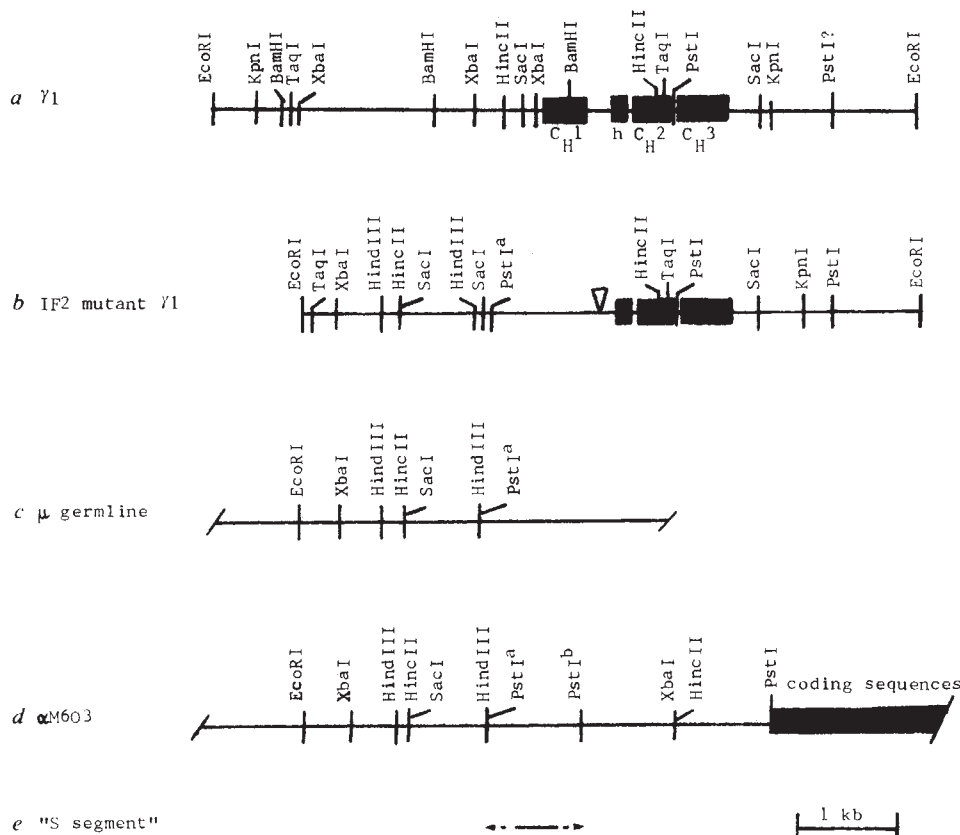


Fig. 3 Restriction maps of various DNA segments associated with mouse heavy-chain genes. The restriction maps shown are the wild-type P3 $\gamma 1$ gene^{9,10} (a), the IF2 mutant $\gamma 1$ gene (b), a segment of the $C\mu$ gene^{24,30} (c) and the α gene of M603 myeloma cells²⁴ (d). The restriction map of the IF2 $\gamma 1$ gene was constructed from Southern filter hybridization data of digests of $\gamma M14$ DNA, and from restriction analysis of the isolated 6.0 RI fragment. The α gene from M603 cells is known to have introns; we do not show them in d. e Shows the region in which the switch leading to the M603 α gene took place (the S segment).

introduces severe constraints on models implicating secondary structure of introns in splicing events, and highlights the importance of intron sequences near the splicing points¹⁶⁻¹⁸.

The IF2 deletion and human HCD proteins

IF2 is analogous to HCD proteins where the deletion involves a single exon^{7,8}. In other HCD proteins, deletions involve part or all of the V region in addition to the CH1 domain⁷. The amino terminus of HCD proteins may or may not be recognizable as V region. Still other HCD protein deletions do not involve the V-C junction at all; in some cases, only the hinge is deleted, in others CH3 is apparently missing⁷. The HCD proteins are thus a heterogeneous group, and are probably the result of several different types of mutation. Some are likely to be the result of nonsense mutations^{1,7}, and mutations in RNA splicing signals have been hypothesized to be involved in HCD protein variants¹⁹. We have shown that one such protein, the IF2 heavy chain, is the result of a DNA deletion. Below we propose that others, especially those centering on the V-C junction, will also reflect deletions at the gene level. Many of these deletions may result from errors in the recognition of class switch signals.

The heavy-chain switch

Early in the immune response, the dominant heavy-chain class is μ ; later the dominant class becomes γ or perhaps α . This switch pattern in whole animals can also be demonstrated in single cells or in clones of single cells, although the exact sequence and restrictions of the class switch are somewhat controversial²⁰⁻²³. It is now believed that the molecular events in commitment to a single VH gene and in the class switch are as follows: a single VH gene is first expressed by an integration event to a putative JH fragment associated with $C\mu$ (ref. 24), an event analogous to the one observed in the joining of VL and JL in light chains²⁵⁻²⁸, for the putative signal sequences adjacent to VH genes are

homologous to those in VL genes²⁹. This leads to VHC μ expression. The class switch requires a second, different form of rearrangement, involving a different segment of DNA believed to lie in the intervening sequence between VH and $C\mu$ (refs 11, 24, 30). Recent hybridization analysis of DNA from cells making various classes of heavy chains^{11,31,32} support the hypothesis proposed by Honjo and Kataoka³³ that the class switch occurs by deletion events involving the exons and introns of all CH genes between the expressed V region and the new CH gene to be expressed.

Davis and colleagues²⁴ have presented evidence that in an α myeloma about half of the intron between VH and $C\alpha$ is derived from the germ-line μ gene; the other half is derived from the germ-line $C\alpha$ gene. The position of this sequence change occurs between the sites labelled $PstI^a$ and $PstI^b$ in Fig. 3c, d, as $PstI^a$ and all sites to the left are found in the $C\mu$ gene, whereas $PstI^b$ and all sites to the right of $PstI^b$ are found in the germ-line $C\alpha$ gene²⁴. A similar situation has been described in an expressed $\gamma 1$ gene³⁰. In both cases, the switch seems to have taken place within the DNA segment designated S in Fig. 3e. It is reasonable to suggest this region as being crucial in the heavy-chain switch.

The S segment in $\gamma M14$ DNA

The restriction map of the IF2 gene to the left of the deletion boundary (Fig. 3b) corresponds to a part of the germ-line $C\mu$ gene and the homologous part of the expressed α M603 gene (Fig. 3c, d). In other words, the intron fragment of the $C\mu$ germ-line gene which is retained during the deletion leading to the class switch is part of the $\gamma M14$ DNA we have cloned. We were therefore in a position to study the sequences which may be involved in the heavy-chain switch.

Part of the $\gamma M14$ sequence which contains the 'S segment' is shown in Fig. 4. Immediately at the site of the IF2 deletion (at the bottom of Fig. 4), tandemly repeated homologous sequences begin. The repeat unit is 49 base pairs long and is repeated

10 1/3 times. No two of the repeated sequences are identical, varying from each other by 1 to 14 residues. This set is preceded by 11 unrelated nucleotides and then by a second set of repeats of a basic unit beginning with GGGGT followed by two to four GAGCT pentanucleotides. Towards the left (the V region), the repeat drifts away from the basic unit and the number of GAGCT subunits becomes more variable. There is a total of about 38 repeats of such elements with an average content of two to three GAGCT subunits. The divergence is such that no significant homology is retained within 100 bases of *Pst*I^a.

In a general sense, tandemly repeated sequences would be ideal for promotion of DNA deletions like those that take place in the heavy-chain switch. A simple hypothesis could then be that each heavy-chain C gene is preceded by homologous S segments containing tandemly repeated units. Recombination by pairing between the repeats (for example, from a μ gene and a γ gene) would result in deletion of all the sequences between

them. This hypothesis predicts (1) the presence of at least one S segment for each C region in the germ line, and (2) the loss of one germ-line S segment for each CH gene lost during a switching event. To test this hypothesis we have used the 1.8 *Pst* fragment, which also contains the γ 1 hinge and CH2 coding sequences, as a probe for the S segment in *Eco*RI-digested kidney and myeloma DNAs (Fig. 5). The *Eco*RI fragments containing the γ 2a (ref. 11), γ 2b (ref. 34) and γ 1 (refs 9, 10) coding sequences, which hybridize to the γ 1 coding sequences in 1.8 *Pst*, are detected in kidney DNA (slot 6). In addition, at least five *Eco*RI fragments from kidney DNA hybridize to the 1.8 *Pst* fragment (bands a, b, d, e, f) but not to the γ 1 coding sequences (results not shown, see also ref. 9). This result agrees with prediction (1) of multiple germ-line S segments. In agreement with prediction (2), at least three of these fragments are lost as one follows the expression of heavy chains. These are b, a and d as one follows the expected progression kidney- μ - γ 1- γ 2b- γ 2a- α

70 AGACTCATAAAGCTGGCTGAGCTCAAAATTAAGGGAACAAGCTTGAGAGCCCTACTAAGCGAGGCTCTAAAA
140 AGCATGGCTTGAGCTGAGCTGGCTGGCTTCTCTGAGCGCTTCTAAAAATGGCTAAAGCTGAGGTGATTAC
210 TCTGAGCTTAAGCTAAAGCTGGGCTTGAGCCAAATGAAGTACACTGTAACTGAAGCTGAGCTGGGCG
280 GCTAAGCTAAAGCTAGGCTGGCTTAACCGAGATGAGCCAAAGTGAATCAAGCTCATTAATCTAGCTTGAA
350 TAGAGCTAAAGCTCTACTGCTACACTGGAGCTCTCTGAGCTGAGCTGAGCT

402 GGGGTGAGCTCAGCTATGCTACGCTCTCTT

432 GGGGTGAGCTCATCTGAGCTGAGCTGAGCT

462 AGGGTGAGCTGGAGCTGAGCT

482 GGGGTGAGCTGAGCTGAGCT

502 GGGGTAAGCT

(This type of pattern is repeated ca. 24 more times)

ca. 850 GGGGTGAGCTGAGCTGAGCT

870 ————— GAGCT

895 —————

915 —————

935 ————— ()

950 —————

970 —————

990 ————— A

————— C

1010 —————

1030 GATTACTTATA

tandem repeat:

20 residues x 9

1041 GGGAGCCAGGACAGCTGGAAGTCTGGTGAGCCAGGACAGGAGCTCCAG

1090 ————— A T ————— A

1139 — C ————— A ————— G C T ————— A T —————

1188 — T T ————— C —————

1237 — C —————

1286 — C ————— A T —————

1335 — A ————— A ————— T ————— T T —————

1384 ————— A C ————— T T ————— T ————— A ————— A —————

1433 ————— A ————— A —————

1482 — C — T — A ————— A ————— C ————— T ————— T ————— T T —————

1531 ————— C T C T A C T C T C A A A C C C A G T A A C A C A T A G C T T C T C C A C A T G C C A G G A T

site of deletion

ValProArgAsp

intron

hinge

Fig. 4 Tandemly repeated sequences near the site of the IF2 deletion. The numbers indicate the distance in base pairs from *Pst*I^a (Figs 2b, 3). In residues 70–401, the underlined nucleotides show homology (80% or more) to the pentanucleotide GAGCT. In residues 402–511, there is a repetitive pattern beginning with GGGGT followed by one to five GAGCTs, or some close derivative of it. These patterns can be seen on the upper half of sequencing gels; we estimate that this pattern is repeated 24 more times (residues 511–850). In residues 850–1,029, this repetitive unit has become more or less exact, except for variations in the number of GAGCT pentanucleotides. To emphasize the more exact repeat unit, residues 850–1,029 are enclosed by dotted lines, and solid lines indicate concordance with the sequence at the top line of the box. After a short sequence (residues 1,030–1,040), a new tandem repeat appears, extending from residues 1,041 to 1,545 (the site of the IF2 deletion). Again, the repeated sequences are enclosed within dotted lines and solid lines indicate concordance with the sequence at the top line of the box. The repeated units in residues 402–1,040 and those in residues 1,041–1,545 are apparently unrelated. These sequences were determined as described in Fig. 2. As the sequence from residues 444 to 1,040 was determined on one DNA strand only, we judge it to be 95% accurate.

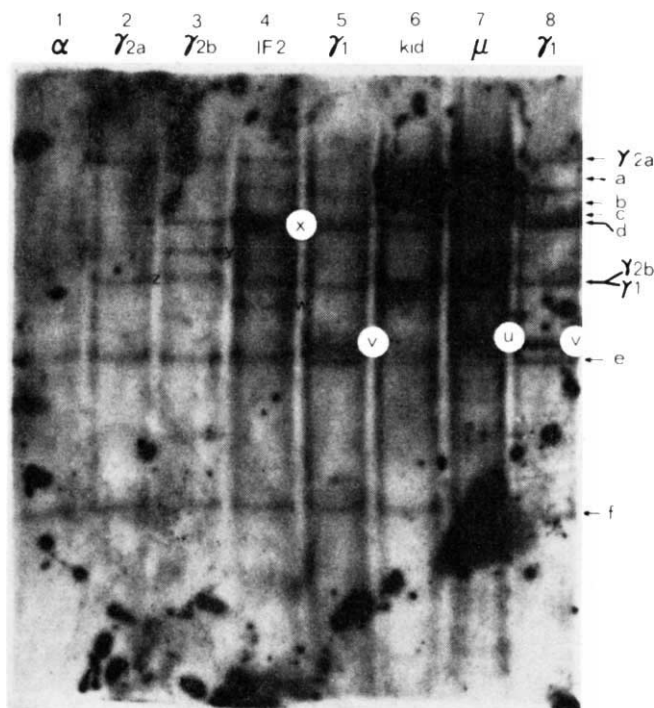


Fig. 5 Arrangement and rearrangement of tandemly repeated and associated sequences in DNA from myelomas secreting various classes of immunoglobulin. DNAs from kidney cells and various myelomas were digested with *Eco*RI and analysed by the Southern filter hybridization technique¹¹. Hybridization to pγ1/IF2P.1.8 was in 6×SSC (no dextran sulphate). Slot 1, MOPC 315 DNA (α heavy-chain producer); slot 2, P1 DNA (γ2a heavy-chain producer); slot 3, MPC 11 DNA (γ2b heavy-chain producer); slot 4, IF2 DNA (γ1 mutant heavy-chain producer); slot 5, X63 DNA (γ1 heavy-chain producer); slot 6, kidney DNA; slot 7, MOPC 104E DNA (μ heavy-chain producer); slot 8, NSII/1 DNA (γ1 heavy-chain producer); the NSII/1 heavy chain is smaller than the parental X63 γ1 heavy chain; the nature of the defect is not known³⁵. All DNAs were prepared from tissue culture cells except for 104E (slot 7) which was from ascites cells. Infiltration by lymphoid and non-lymphoid tissue could account for minor contaminants. Bands labelled γ1, γ2b and γ2a contain coding sequences which cross-hybridize to the cDNA clone pγ/A5 (see also ref. 11).

Note that in the γ2b producer (slot 3), the γ2b band has, albeit slightly, shifted position. The switch to γ2b production deletes the γ1 gene^{11,31-33}. The γ2b sequences may be rearranged to give an *Eco*RI fragment of a slightly different size. This band and all others in the γ2b/γ1 position are different from band z present in the γ2a producer (slot 2). Band z does not hybridize to the γ1 cDNA sequences (data not shown); we presume that this contains the rearranged S segment.

(refs 11, 30–32). In addition, for most of the myelomas tested, new fragments hybridizing to the noncoding part of the 1.8 *Pst* probe can be identified (bands u, v, y and z). This is presumably the result of sequence rearrangement in the environment of S segments during the switch. To describe it schematically, let us take the sequence A-B-C-B-D-B-E. Deletion of B-C would yield A-B-D-B-E, and would involve a change in two fragments containing B. A-B-C and C-B-D are lost, D-B-E is retained, and a new fragment, A-B-D, is formed. Specifically, we suggest that bands u, v, y and z are the rearranged forms of S present when μ, γ1, γ2b and γ2a (respectively) are expressed. We have not rigorously shown that u and v are different, but we have shown that the z component of γ2a and γ2b are indeed different. The latter cross-hybridizes with the γ1 coding sequences whereas the former does not (results not shown, see also ref. 9). This highlights the shortcomings of this type of analysis. Fragments may exist which are not detected (too large or too small) or are hidden under other hybridizing fragments. For example, we have no good candidates for the Cγ3 and Cα germ-line S

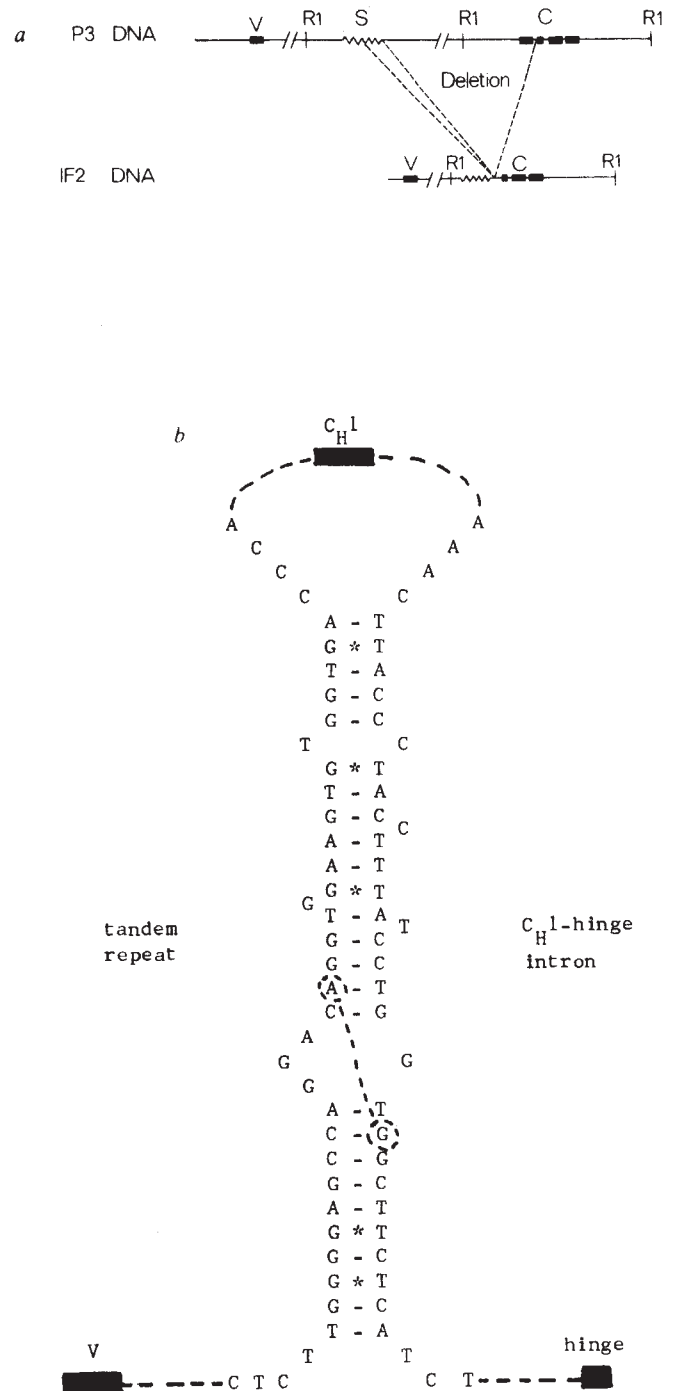


Fig. 6 a, Generation of the IF2 mutant. The hypothesized structures of P3 γ1 gene and of IF2 γ1 gene are shown, with coding sequences in darkened boxes and the S segment in a zig-zag line. The dotted lines denote the extent of the IF2 deletion, which could begin in the S segment or at its right end. b, A putative hairpin loop at the site of the IF2 deletion. The left-hand side of this structure below the circled A was constructed using the sequence of the IF2 gene to the left of the deletion (Fig. 4, residues 1,525–1,543). The left-hand side above the circled A was constructed from the consensus sequence of the 49-base tandem repeat (which is represented by residues 1,054–1,073, Fig. 4). The right-hand side comes from the Cγ1 sequence¹⁰ near the site of the IF2 deletion (see also Fig. 2d). Ligation of the circled A and G would result in the sequence at the site of the deletion in the IF2 gene (Fig. 2) and deletion of the sequences above these two residues. G + T base pairs are shown by an asterisk; in the other DNA strand (not shown) these would be unpaired A + C.

segments. On the other hand, there are other components (for example, band c) for which we find no good explanation. Two germ-line fragments (e and f) hybridizing to the 1.8 *Pst* fragment, but not to the $\gamma 1$ cDNA clone, persist even in the α producer. These fragments may correspond to the other heavy chains ϵ and/or δ , to a hitherto unidentified heavy chain, or to repeats of a similar sequence unrelated to immunoglobulins.

From the experiment shown in Fig. 5, the minimum size of the IF2 deletion can be calculated. IF2 DNA (slot 4) shows two new bands, x and w, and lacks the rearranged band v. Band x is the product of partial *Eco*RI digestion we have cloned. Presumably, the IF2 deletion resulted in fusion of band v and the $\gamma 1$ coding sequences to yield band w (the 6.0 RI fragment). Thus, the size of the IF2 deletion is 5.5 kilobases [6.8 kilobases ($\gamma 1$ coding sequences) + 4.7 kilobases (band v) - 6.0 kilobases (band w)], assuming no *Eco*RI fragments between band v and the fragment containing the $\gamma 1$ coding sequences.

We would like to argue that the signals for the class switch are based on the tandem repeats described above. As we have discussed, the switch occurs in or near the S fragment, which is mostly composed of these tandem repeats. Further evidence for involvement of the tandem repeats is provided by a comparison of the work of Kataoka *et al.*³⁰ and the one described here. Based on the restriction maps shown in Fig. 3, one can locate in their work an S segment which included the putative switch point. A close inspection of their sequence shows the presence of four sequential pseudo-repeats homologous to the 49-base pair repeat shown in Fig. 4 (starting 14 residues from the putative switch point). Preceding these sequences is a long sequence about 10% of which is GAGCT.

The difference between the sequences described by us and those described by Kataoka *et al.*³⁰ could have trivial sources, such as drift during tumour propagation. The difference could be a consequence of the deletion which gave rise to IF2. We prefer a third alternative, which is that these differences are a consequence of the switch process itself. The precise point of the switch could vary along the extended tandemly repeated sequences in both the donor gene (for example, μ) and the acceptor gene (for example, γ)^{24,30}. Therefore, two rearranged $\gamma 1$ genes could have different S sequences, depending on how the donor and acceptor S sequences aligned, and where the recombinational event took place. Also, the S sequences in $\gamma 1$ genes might vary depending on whether or not the V regions went through a VH-C γ 3 intermediate.

HCD proteins in general, and IF2 in particular, have been implicated with DNA rearrangements for several years. Specifically, it has been proposed that signals for DNA rearrangements were duplicated along with primordial CH

domains during the evolution of modern CH genes^{3,5}. The signals preceding each CH gene retained their function while those between CH domains drifted. Some HCD deletions could result from the illegitimate use of the ancestral homologue within a single CH gene, as opposed to legitimate use of switch signals between CH genes.

With this in mind, we searched for homology between S-segment sequences, which we believe to be the switch signals, and sequences in the normal $\gamma 1$ gene^{9,10}. This search resulted in a hairpin loop structure at the site of the deletion (Fig. 6). This hypothetical structure was formed between a consensus sequence of the 49-base repeat and the sequence of the $\gamma 1$ germ-line gene at the site of the deletion. The sequence of the IF2 $\gamma 1$ gene at the deletion results from the ligation of the circled A and G (see also Fig. 2c). The sequences above these two residues would represent the deletion. Such hairpin loop structures can only be speculative. The free energy of the structure shown in Fig. 6b is +3.4 kcal mol⁻¹ (refs 36, 37), corresponding to a melting temperature of 30 °C. This value can be compared with those of similar structures. For example, the proposed hairpin structure for κ V-J joining^{27,28} has a free energy of about -19 kcal mol⁻¹ (T_m = 67 °C). Obviously, for a low frequency event such as a mutation, one expects relatively unstable structures.

In conclusion, we suggest that the deletion which gave rise to the IF2 mutant, and also the deletions for similar HCD proteins⁵, were the result of illegitimate use of switch signals. Whether the proposed hairpin loop formation, or other mechanisms such as sister chromatid exchange³⁸, represent a model for the physiological switch remains to be seen.

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After submission of our article, we received a copy of manuscript by Sakano *et al.* (see accompanying article³⁹). Our sequence (Fig. 4) up to residue 448 is identical (except for nine bases) to their sequence of the germ-line $C\mu$ gene between residues 1,114 and 1,490. After residue 448, however, the two sequences differ drastically. It seems, therefore, that in the P3 lineage the switch from the $C\mu$ gene occurred to the right of this point. Apparently, in the three cases so far described, the switch has taken place at three different sites. Furthermore, tandemly repeated units in the sequences of Sakano *et al.*, Kataoka *et al.* and ourselves are in the same polarity, implicating homologous pairing rather than hairpin loop formation in the DNA exchange mechanism associated with the switch.

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Two types of somatic recombination are necessary for the generation of complete immunoglobulin heavy-chain genes

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At least two types of somatic recombination are necessary for the generation of a complete immunoglobulin $\gamma 2b$ gene from germ-line DNA sequences. The first type of recombination consists of the assembly of three separate DNA segments, each encoding a different part of the variable region. The second type of recombination replaces the exons coding for the constant region of the μ chain with those coding for the same region of the $\gamma 2b$ chain. The DNA sequencing studies suggest that the two types of recombination operate by different mechanisms.

COMPLETE, active immunoglobulin genes are created by somatic recombination that occurs during the differentiation of lymphocyte precursor cells¹⁻³. The organization of the gene sequences before and after somatic recombination has been studied extensively for both λ - and κ -type light chains in the mouse³⁻¹⁰. These studies established that in the embryonic genome, the major portion of the conventionally defined variable (V) region is encoded in a DNA segment (V DNA) that is located some distance away from a DNA segment (J DNA) coding for the rest of the V region. The J DNA segment has been mapped a few kilobases upstream of (that is, 5' side, with respect to the direction of transcription) the single-copy DNA segment (C DNA) coding for the constant (C) region. In the myeloma cells in which the light-chain gene in question is active, the V DNA segment and the J DNA segment are contiguous as a consequence of a recombination that apparently accompanies deletion of the DNA sequence occurring between the V and J DNA segments in the germ-line genome⁷. The entire region of the chromosome containing the V DNA segment, J DNA segment, J-C intron and C DNA segment is transcribed into a single RNA molecule from which a mature messenger RNA is generated by RNA splicing^{11,12}. The V-J joining step is considered to be a key event in the activation of the immunoglobulin gene. It may also contribute to the increase of the organism's capacity to synthesize a large number of different antibodies^{7,13-15}.

As the heavy-chain polypeptide is also composed of a V and C region, one might assume that what has been established at the DNA level for the light chains would also be true for heavy chains. However, the expression of heavy chains has some features which make it considerably more complex than that of light chains. For instance, unlike the light chains, there are in the mouse at least eight different C regions, each of which seems to share the same set of V regions, that is, a given V gene can be expressed with more than one heavy-chain class or subclass. In addition, it seems that, as the lymphocytes differentiate, the C region of the heavy chain synthesized switches from one class to another without alteration of the V region¹⁶⁻¹⁸.

Recently, we reported on the structure of the complete $\gamma 2b$ gene isolated from a myeloma, MOPC141, an IgG2b secretor¹⁹. These studies showed that, unlike the light-chain genes, at least two recombinations are necessary to generate the heavy-chain gene. One of them occurs at or near the 3' end of the V DNA segment and in the vicinity of the J DNA segment. In contrast to the light-chain genes, where the J DNA segments are located in the 5'-flanking regions of their respective C genes, in the heavy-chain gene system the J DNA used for the MOPC141 $\gamma 2b$ gene does not lie in the 5'-flanking region of the germ-line $C\gamma 2b$ gene,

but is located in the 5'-flanking region of the germ-line $C\mu$ gene. This recombination between V DNA and J DNA near the $C\mu$ gene probably allowed the μ gene to be activated in the precursor B cells to MOPC141 myeloma. The second recombination occurs between a pair of sites, one located between the J DNA segment and the $C\mu$ gene and the other in the 5'-flanking sequence of the $C\gamma 2b$ gene, and replaces the $C\mu$ -coding exons with the $C\gamma 2b$ exons. This recombination is referred to as 'switch recombination', for it seems to be a key event preceding the heavy-chain switch. A similar gene structure was also observed for a complete α -chain gene²⁰ and for a $\gamma 1$ -chain gene²¹.

We report here on the two types of recombination, studied by DNA sequencing. The sequences around the V-J joining and the switch recombination sites have different features, suggesting that at least two distinct enzyme systems are involved in the generation of the active immunoglobulin $\gamma 2b$ gene. In addition, sequence analysis of the V DNA of MOPC141 and its germ-line components, the embryonic V DNA and J DNA, revealed that the third hypervariable region is encoded in a separate DNA segment(s) in the germ-line genome.

Mouse DNA inserts in the DNA clones used in this study are listed in Fig. 1. The homology among these DNA fragments has been determined by heteroduplex analysis and restriction enzyme mapping. The coding regions were mapped by R-loop analysis using mRNAs from MOPC141 ($\gamma 2b$) or MOPC104E (μ). Isolation and electron microscopic characterization of clones M141-P21 (complete $\gamma 2b$), MEP203 (embryonic J plus $C\mu$) and MEP3 (embryonic $C\gamma 2b$) have been described elsewhere¹⁹. The isolation of the embryonic V-gene clone, PJ14, is described below.

Identification of J DNA for MOPC141

We have previously identified the putative V-J joining site on the complete $\gamma 2b$ gene clone (M141-P21) and on an embryonic $C\mu$ gene clone (MEP203) by heteroduplex analysis and R-loop mapping¹⁹. The electron microscopic studies indicated that the putative J DNA for the MOPC141 heavy-chain gene lies 3.6 kilobases 5' to the $C\gamma 2b$ gene on M141-P21 and 5 kilobases 5' to the $C\mu$ gene on MEP203. To analyse the structural features of heavy-chain V-J joining sites, we determined the nucleotide sequences of the appropriate regions of M141-P21 and MEP203. Although the amino acid sequence of the MOPC141 heavy chain is unknown, identification of the J DNA segment coding for this chain was possible because J-peptide sequences are highly conserved. As shown in Fig. 2, M141-P21 carries a DNA sequence in the vicinity of the putative V-J joining sites which can encode a peptide that is identical to the J peptides of

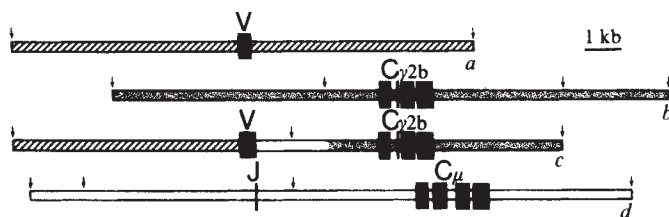


Fig. 1 Mouse DNA inserts in the clones used in the study. Embryonic V_{M141} gene clone PJ14 (a), embryonic $C\gamma 2b$ gene clone MEP3 (b), complete $\gamma 2b$ gene clone M141-P21 (abbreviated to M141) (c) and embryonic $C\mu$ gene clone MEP203 (d) are shown. Arrows indicate *EcoRI* cleavage sites. Filled boxes are exons identified by R-loop mapping. The regions of homology among the four DNA clones as determined by heteroduplex analysis are indicated by bars of different types of shading.

MOPC21 and MOPC173 heavy chains (see Table 1). We also analysed the MEP203 insert to determine whether the same J DNA segment is present in the expected position. Our previous study on clones MEP203 and M141-P21 suggested that the J_{M141} DNA segment and the sequence that follows this segment are derived from a region 5' to the $C\mu$ gene. In fact, the sequence of MEP203 showed that the J DNA segment and its 3' non-coding sequence found on M141-P21 are also present in the region about 5 kilobases 5' to the $C\mu$ gene on MEP203 (Fig. 2). Comparison of these two J_{M141} DNA sequences, one on M141-P21 and the other on MEP203, indicates that the germ-line J_{M141} DNA can encode the M141 J peptide starting with the second nucleotide of the Leu codon at position 112.

In $\gamma 2b$ heavy-chain peptides, the boundary of V and C regions has been thought to be around Ala 126 (ref. 22). The J_{M141} DNA segment can fully encode Ser 125, but only partially Ala 126. We found a triplet, GGT, instead of the Ala codon GCN. Because, in almost all cases studied thus far, an intron starts with the doublet GT (ref. 23), we tentatively conclude that the coding of the J_{M141} DNA ends with the first letter G in the triplet GGT.

Four heavy-chain J DNA segments around 8 kilobases 5' to the germ-line $C\mu$ gene

Previous studies showed that most, if not all, κ -light-chain J DNAs are tightly linked^{7,13}. To examine whether the same applies to the heavy-chain J DNA segments, we isolated DNA clones containing the rearranged V gene from myelomas MOPC603, MOPC315, HOPC8 and MOPC173 and analysed the heteroduplexes formed between each of these DNA clones and the embryonic μ DNA clone MEP203. It is expected that the divergency point observed on a Y-shaped heteroduplex molecule corresponds to the J DNA segment used for expression in the respective myeloma cells. In this way, we mapped four different J DNA segments in the region 5' to the $C\mu$ gene, at 1.1 (MOPC173-II J), 1.6 (HOPC8-II J), 1.9 (MOPC315 J) and 2.2 (MOPC603 J) kilobases, 5' to the *EcoRI* site located 3.5 kilobases from $C\mu$ DNA on MEP203. As MEP203 carries a 3-kilobase deletion at 1.5 kilobases 5' to the $C\mu$ gene (see below), the *EcoRI* site is 6.5 kilobases from the $C\mu$ gene on the mouse chromosome. Using μ -chain mRNA from MOPC104E in R-loop analysis, we also identified the J_{M104E} DNA segment at 2.3 kilobases 5' to the *EcoRI* site (data not shown). As the MOPC104E μ chain and the MOPC603 α chain seem to have the same J peptide (Table 1), this J DNA segment is probably the same as that detected by heteroduplex analysis using the MOPC603-derived V-gene clone.

Figures 3Aa and 4 show the restriction map and the DNA sequence of the 1.5-kilobase J-rich region, respectively. So far, 22 heavy chains have been sequenced in the J-peptide regions.

They can be classified into four different groups according to the amino acid sequences of the J peptides (Table 1). Comparison of these J-peptide sequences with the DNA sequence allowed identification of the corresponding four J DNA segments. These four segments can account for all heavy-chain J peptides sequenced so far (see Table 1) and, therefore, seem to encode the J peptides of all different classes or subclasses. We designate these J DNAs as J_{H1} , J_{H2} , J_{H3} and J_{H4} , from 5' towards 3'. No additional J-like DNA sequence has been observed in the 1.5-kilobase region sequenced thus far. There is one unexpressed J DNA, J_{K3} (ref. 7), in a κ -light-chain gene, and there may also be some in the heavy-chain gene.

Isolation of the germ-line V-gene clone for the MOPC141 heavy chain

To determine the recombination site on the germ-line V gene, we attempted to identify and clone the *EcoRI* DNA fragment carrying the germ-line V gene for the MOPC141 heavy chain. First, we analysed embryonic DNA by Southern hybridization using the V DNA probe purified from M141-P21. This probe is a 257-base pair *AvaII-HgaI* fragment encoding residues 9–94 of the M141 heavy chain (Fig. 3Ab). In the *EcoRI* digest of mouse embryo DNA, about 10 hybridizable bands were detected (data not shown). Detection of 5–10 bands by a single V-gene probe has previously been reported for both κ light chains^{5,9} and heavy chains^{24–26}. This is due to cross-hybridization among a subset of germ-line V genes that have presumably diverged more recently. As the length of the 5'-noncoding region of the V_H gene on M141-P21 is 6.5 kilobases, the size of the *EcoRI* fragment carrying the germ-line V_H DNA for the M141 heavy chain should be larger than 6.5 kilobases. We separated the *EcoRI* digest of embryonic DNA on a preparative agarose gel, eluted the DNA from the 6.5–23-kilobase region and used it for DNA cloning. Using the V-region probe, we isolated some 30 clones from 2 separate experiments and classified them into 5 different types according to the size of the *EcoRI* insert. Heteroduplex molecules formed between the clones of each type and M141-P21, a complete $\gamma 2b$ gene clone, were examined by electron microscopy.

Although the *EcoRI* inserts of these clones were all hybridizable with the V-gene probe, only the clones containing a 14-kilobase insert formed Y-shaped heteroduplex molecules with M141-P21, in which the homology extended from the 5' *EcoRI* end to a site 6.9 kilobases away from it. R-loop analysis using the MOPC141 $\gamma 2b$ mRNA indicated that the V-coding DNA segments map at or around the heteroduplex fork point. We further analysed one of these 14-kilobase clones, PJ14, by restriction mapping and DNA sequencing. Evidence that the 14-kilobase clone contains the germ-line V gene for the MOPC141 $\gamma 2b$ chain is obtained from restriction enzyme maps. The cleavage sites for *BamHI*, *KpnI*, *XbaI*, *SacI* and *HindIII* in the 5'-flanking region of the V gene on M141-P21 were all found at the corresponding positions on the embryonic V-gene clone, PJ14 (data not shown).

The third hypervariable region is encoded separately

The DNA sequences around the V-coding regions of PJ14 and M141-P21 are shown in Fig. 2. The sequences include a 46-base pair exon presumably coding for the hydrophobic signal peptide, an 81-base pair intron, the entire V-gene exon and the 3'-flanking regions. As in all κ - and λ -chain genes studied so far (refs 4, 6, 8 and G. Heinrich, unpublished results), the V coding begins with the residue at position -4 within the signal peptide. Also, as in the light-chain genes, the coding by the embryonic clone PJ14 ends prematurely with Ser 97, whereas that of the myeloma clone, as mentioned above, continues up to Ser 125. In the 0.6-kilobase stretch of DNA starting with the *HindIII* site in the 5'-noncoding region and ending with the Ser 97 codon, the

Table 1 Amino acid sequences of mouse immunoglobulin heavy chains around the carboxyl ends of the V regions

Myeloma	End of V	HV3	J	J DNA
M104E	... YYCAR	DY	d W Y F D V W G A G T T V T V S S	
J558	... YYCAR	D	r Y - - - - -	
T15	... YYCAR	DYYGS	s Y - - - - -	
M603	... YYCAR	NYYGST	- - - - -	
M167	... YYCTR	DADYGD SYF	g - - - - -	J _{H1}
T601	... YYCAR	LGYY	g - - - - -	
S107	... YYCAR	DYYGS	s Y - - - - -	
H8	... YYCAR	DYYGN	s Y - - - - -	
M511	... YYCAR	DGDYGS	s Y - - - - -	
M315	... YYCAG	DNDHL	Y F D Y W G Q G T T L T V S S	
X24	... YYCAR	LGYYG	- - - - -	J _{H2}
Hdex4	... YYCAR	DK	n - - - - -	
Hdex5	... YYCAR	DS	n - - - - -	
A4	... YYCTT		s W F A Y W G W G T L V T V S A	
E109	... HYCTT		g - - - - -	
U61	... YYCTT		g - - - - -	
A47N	... YYCST		g - - - - - P - - -	J _{H3}
X44	... YYCAR	LHYYGYA	- - - - -	
J539	... YYCAR	LHYYGYN	- - - - -	
M141	... YYCAS	VSIYYYGRSDKYFT	I D Y W G W G T S V T V S S	
M21	... YYCAR	HGNYPW	Y A M - - - - -	J _{H4}
M173	... YYCAR	SP	Y Y A M - - - - -	

Amino acid sequences of 22 mouse heavy chains^{27,32,34-40} for residues 90-113 (numbering is after Kabat *et al.*²²) are shown by one-letter codes⁴¹. According to the J-region peptides, the chains are classified into four groups. The amino acid sequence of the MOPC141 γ 2b chain was deduced from the nucleotide sequences determined in this study. The first amino acid residues in the J peptides are shown in small letters whenever the first letters of their codons are not included in the corresponding J DNA segments.

PJ14	AAGCTTGCCTGTGCTTCTTTATCTCTCAGGAACCTCCCCAATGCAAATCAGCCCTCAGGCAGAGGATAAAAGCTCACACAAAGATGAGAAGCCCC
M141	AAGCTTGCCTGTGCTTCTTTATCTCTCAGGAACCTCCCCAATGCAAAGCAGCCCTCAGGCAGAGGATAAAAGCTCACACAAAGATGAGAAGCCCC
PJ14	ATCATCTTCTCATAGAGCCTCCATCAGAGCATGGCTGTCTGGCATTACTCTTCTGCCTGGTAACATTCCTCAAGCTGTAAGTGTCTCAGGGTTTCACAAGAGGGACTAAAGACATGTCAG
M141	ATCATCTTCTCATAGAGCCTCCATCAGAGCATGGCTGTCTGGCATTACTCTTCTGCCTGGTAACATTCCTCAAGCTGTAAGTGTCTCAGGGTTTCACAAGAGGGACTAAAGACATGTCAG
PJ14	CTAATGTGTGACTAATGGTAATGTCACTTGTCACTAGGTATCTTTCCAGGTACAGCTGAAGGAGTCAGGACCTGGCTGGTGGCCCTCAGAGCCTGTCCATCAGATGCACCGTC
M141	CTAATGTGTGACTAATGGTAATGTCACTTGTCACTAGGTATCTTTCCAGGTACAGCTGAAGGAGTCAGGACCTGGCTGGTGGCCCTCAGAGCCTGTCCATCAGATGCACCGTC
PJ14	SerGlyPheSerLeuThrGlyThrGlyValAsnTrpValArgGlnProProGlyLysGlyLeuGluTrpLeuGlyMetIleTrpGlyAspGlySerThrAspTyrAsnSerAlaLeuLys
M141	TCAGGGTTCTCATTAAACCGGCTATGGTCTAACTGGGTCGCCAGCCTCCAGGAAGGGTCTGGAGTGGCTGGGAATGATATGGGGTATGGAAGCACAGACTATAATTCAGCTCTCAAA
PJ14	SerGlyPheSerLeuThrGlyThrGlyValAsnTrpValArgGlnProProGlyLysGlyLeuGluTrpLeuGlyThrIleTrpGlyAsnGlySerThrAspTyrAsnSerThrLeuLys
M141	TCAGGATTCTCATTAAACCGGCTATGGTGTGAATCTGGGTCGCCAGCCTCCAGGAAGGGTCTGGAGTGGCTGGGAACGATATGGGGTATGGAAGCACAGACTATAATTCAGCTCTCAAA
PJ14	SerArgLeuSerIleSerLysAspAsnSerLysSerGlnValPheLysLysMetAsnSerLeuGlnThrAspAspThrAlaArgTyrTyrCysAlaSer
M141	TCCAGACTGAGCATCAGCAAGGACAACCTCCAAGAGCAAGTTTCTTAAAAATGAACAGTCTGCAAACTGATGACACAGCCAGGTACTACTGTGCCAGAGACACAGTGAGGGAAGTCCAA
PJ14	SerArgLeuThrIleThrLysAspAsnSerLysSerGlnValPheLysLysMetAsnSerLeuGlnThrAspAspThrAlaArgTyrTyrCysAlaSer
M141	TCCAGACTGACCATCACCAAGGACAACCTCCAAGAGCAAGTTTCTTAAAAATGAACAGTCTGCAAACTGATGACACAGCCAGGTACTACTGTGCCAGCGTCTCAATTATTAATCACTACGGT
PJ14	TGTGAGCCTGCACAAATACCTCTCTGCAGGGATGATCACACCAGCAGGGGGCGCTGAGGACCCAAAGACTT
M141	ArgSerAspLysTyrPheThrLeuAspTyrTrpGlyGlnGlyThrSerValThrValSerSer
MEP203	CCTAGGCACAAATACCTTCACTTGGACTACTGGGGTCAAGGAACCTCAGTCTCTCTCAGGTAAGAATGGCTCTCCAGGTCTTTATTTTAACTTTTGTATGGAGTTTCTCT

Fig. 2 Nucleotide sequences of MOPC141 V_H DNA (M141), its embryonic V_H DNA (PJ14) and the embryonic J DNA (J₄) coding for the J peptide of the MOPC141 γ 2b chain (MEP203). Sequencing strategy is shown in Fig. 3A. Sequence determination was carried out according to the method of Maxam and Gilbert⁴³. The amino acid sequence of the complete MOPC141 V region predicted by the nucleotide sequence of clone M141 is shown in italics. The amino acid sequences predicted by the germ-line V DNA segment in clone PJ14 and the J₄ DNA segment in clone MEP203 are also shown. The nucleotide differences observed between the two clones, M141 and PJ14, are shown by asterisks. Vertical lines indicate the possible recombination sites. The two conserved sequences in the 3'-flanking region of the embryonic V DNA are underlined. HV1, HV2 and HV3 designate the three hypervariable regions.

Figure 2 also shows the DNA sequence around the J_{M141} DNA segment of the embryonic clone MEP203. As described before, coding begins within the Leu 112 triplet and ends with the Ser 125 triplet. It thus seems that the 14-residue peptide comprising the third hypervariable region (HV3) beginning with Val 98 and ending with Thr 111 is encoded in neither the germ-line V gene nor the J DNA segment. This situation is in contrast to that of light-chain genes previously studied⁶⁻⁸, in which no such coding gap was observed. We suggest that the third hypervariable region of this heavy chain is encoded in one or more discrete DNA segments (D DNA segments for 'diversity'²⁷) that lie elsewhere in the germ-line genome. The V, D and J DNA segments must be assembled by recombination to generate the complete V gene present in the myeloma cells.

Close examination of the amino acid sequences around the third hypervariable region and the nucleotide sequences around the 3' end of the germ-line V_H gene and around the 5' end of the J_H DNA segments suggest several interesting features of the DNA joining events. As previously suggested for the light-chain J DNA segments^{7,13-15}, the exact 5' boundary of J_H DNA segments seems to be unfixed (Table 1). For instance, in the case of J_{H4} , the coding of the MOPC141 $\gamma 2b$ chain begins with the second base of the Leu 112 triplet UUG as indicated in Fig. 2. In contrast, coding of the MOPC21 $\gamma 1$ chain and the MOPC173 $\gamma 2a$ chain by the same J_{H4} DNA segment seems to start with a Tyr triplet UAU and another Tyr triplet UAC, respectively. The first letters of the two Tyr triplets are 7 and 10 bases ahead (5' side) of the first base used for the coding of the MOPC141 J region. Similar examples for J_{H1} , J_{H2} and J_{H3} are indicated in Fig. 4 and Table 1.

The DNA sequence of the clone PJ14 demonstrated that coding by the germ-line V_{M141} gene ends with the second letter of the Ser 97 codon AGC (Fig. 2). As shown in Table 1, the amino acid sequences immediately preceding Ser 97 are highly conserved among various V regions. Particularly noteworthy is



Fig. 4 Nucleotide sequence of the 1.4-kilobase region of the embryonic clone MEP203 containing four J_H DNA segments. The sequencing strategy is shown in Fig. 3Aa. The J_H DNA segments are referred to as J_1 , J_2 , J_3 and J_4 from 5' to 3'. The amino acid sequences encoded by these J DNA segments are shown in italics. The sequences closely related to the palindromic heptamer, CACTGTG, and present in front of every J DNA segment, are underlined. The vertical lines indicate determined (solid lines) or predicted (broken lines) sites where coding by the J DNA segments begin in the various heavy-chain genes present in the indicated myelomas. The sites were determined by comparing the J DNA sequences with the sequences of the rearranged V DNA cloned from the respective myeloma. (See Fig. 2 for the M141 $\gamma 2b$ chain gene. The others are based on unpublished sequence data.) We have identified, isolated and sequenced two rearranged V genes, one from HOPC8 and the other from MOPC173. The V regions encoded by these rearranged V genes are different from the published ones, and are therefore referred to as H8-II and M173-II. It is not clear whether these V genes are part of the active heavy-chain genes of the respective myelomas. The putative sites for the heavy-chain genes of S107, M167, M315, A4, J539, M173 and M21 were deduced by comparing the J-peptide amino acid sequence of each heavy chain with the DNA sequence of the corresponding J DNA segment (Table 1).

the universal Cys at position 95. Tyr 93, Tyr 94, Ala 96 and Arg 97 are also well conserved. We therefore believe that the coding by most, if not all, germ-line V genes ends in the immediate vicinity of the 97th triplet. If so, coding gaps of various lengths exist in the majority of the cases listed in Table 1. As suggested above, these residues must be encoded elsewhere in the genome. The gaps in the heavy chains of four myelomas, A4, E109, U61 and A47N, are the shortest: only the first letter of Gly 98 is unaccounted for. Similarly, only several more bases are necessary to code for the HV3 of the J558 α chain. Previous studies on κ -chain genes indicated that the 3' end of a given germ-line V gene may not be precisely fixed and could shift within a range of several nucleotides⁷. If the same applies to the heavy-chain genes, the heavy chains with shorter HV3, such as those of A4, E109, U61 and J558, could be fully encoded by the respective germ-line V gene and the J DNA segment. It thus seems that in some cases the germ-line V DNA segment directly joins with the J DNA segment, as is the case in the light-chain genes. However, it is also possible that the assembly of the V_H always occurs in two steps, but that in some cases the D segments are very short or do not even contribute to protein encoding at all.

In either case, by modulation of the joining sites and by various combinations of these DNA segments, diversity could be generated in the third hypervariable region of heavy-chain molecules. However, the location and number of the D DNA segments in the germ-line genome are unknown. They may be at the 3' end of each of the multiple germ-line V genes or clustered between the V DNA segments and the J DNA segments. In either case we assume that the order of the V, D and J DNA segments in the germ-line genome is the same as in the completely assembled V genes, so that successive joining of the three types of DNA segments is possible by the looping-out of the intervening spacers.

Sequence characteristics around the V and J joining sites

We have previously reported that the five J_K DNA segments and the one J_L DNA segment are preceded by two blocks of short, conserved sequences: a palindromic hexamer interrupted by an AT base pair at the centre of symmetry, CACTGTG, and a nanomer, GGTTTTTGT (ref. 7). Similar sequences are also present 5' to all four J_H DNA segments, although deviation from the basic sequences seems to be slightly higher for the J_H segments than for the J_L segments (Table 2). We also reported previously that germ-line V_K and V_L DNA clones contain inverted complements of the nanomer and heptamer in the 3'-noncoding regions. As shown in Fig. 2 and summarized in Table 2, the same or similar sequences are also present in the equivalent positions of the germ-line V_{H141} gene clone. Based on the fact that these sequences are highly conserved, we propose that they constitute recognition signals for the putative recombinase. The finding of similar sequences near the heavy-chain gene DNA segments strengthens the validity of this hypothesis.

In the case of J_{H3} , two sequences, CACAATG and CAATGTG, are closely related to the basic heptamer sequence, CACTGTG. Similarly, two sequences, TATTGTG and TACTATG, are present near the 5' end of J_{H4} (Fig. 4). In these cases it is not clear which of the two sequences constitutes part of the proposed recognition signal.

The presence of the closely related conserved sequences in the corresponding positions of both light- and heavy-chain germ-line V genes and their J DNA segments suggests that all recombination events necessary for the generation of complete, somatic V genes are carried out by the same or a similar mechanism. We predict that the similar recognition sequences are also present on both sides of the D DNA segments.

An additional feature of the conserved signal sequences is the striking regularity in the length of the spacer between the heptamer and the nanomer. As summarized in Table 2, in almost all cases the spacers are 12 or 23 base pairs long. One exception is one of the two alternative cases of J_{H4} , where the spacer is 31 base pairs long. As a DNA double helix completes a turn every 10.4 base pairs²⁸, the above indicates that the internal boundaries of the heptamer and the nanomer of a given DNA segment are orientated in nearly the same direction relative to the axis of the helix, regardless of the length of the spacer. The spacers of all V_{κ} segments correspond to one turn, and those of $V_{\lambda I}$ and $V_{\lambda II}$ to two turns. The spacer of V_{M141} is two turns long. All J_{κ} spacers are two turns whereas the $J_{\lambda I}$ spacer is one turn. All spacers of the four J_H segments are two turns. The 31-base pair spacer, one of the two alternative spacers of J_{H4} , could be considered as an exceptional three turns. It should be emphasized that although the lengths of the spacers are conserved in a given type of gene segment, their sequences have diverged widely.

A model of the joining enzymes

The above mentioned features around the joining sites suggest some predictions about the nature of the recombinases. In both λ and κ light-chain V-J joinings, one partner in the recombination displays the heptamer and nanomer one turn of the helix apart from the recombinase, and the other displays these sequences two turns apart. We predict that the recombinase contains two DNA-binding proteins: one recognizing the heptamer and nanomer one turn apart, and the other recognizing them two turns apart. As the two partners carry essentially identical recognition sequences, the two DNA-binding proteins would be structurally closely related.

The germ-line V_{M141} gene and all J_H segments show the recognition sequences separated by two-turn spacers. If the D segment contains recognition sequences with one-turn spacers on each side, then V-D and D-J joinings would be mediated by recombinases having components equivalent to those acting on the light-chain genes. In this way, all recombinations leading to the assembly of immunoglobulin V genes would follow a one-turn/two-turn spacer rule.

The two recombinase components hold the two recombining partners together and cut and rejoin the strands in the vicinity of the heptamers. As the recognition sequences of one partner are complementary to those of the other, it is possible that intra-strand base pairing occurs, however transiently, in the enzyme-DNA complex and facilitates the ligation reaction.

This rule prohibits some unwanted recombinations, such as those between V_{λ} and J_{κ} , and V_{κ} and J_{λ} . However, some other unwanted recombinations, such as those between V_H and J_{λ} , D and J_{κ} , and V_{λ} and V_{κ} , could occur. This may be the reason that the three gene families are distributed in three different chromosomes.

The switch recombination sites for the complete $\gamma 2b$ gene of MOPC141

Our previous electron microscopic studies¹⁹ demonstrated that the complete $\gamma 2b$ gene clone M141-P21 shows some homology to the germ-line C_{μ} gene clone MEP203 and some to the germ-line $C_{\gamma 2b}$ gene clone MEP3. As shown in Fig. 1, the homology between clone M141-P21 and MEP203 is 2.4 kilobases long and extends, on M141-P21, from the 3' end of the V DNA segment to a site 1.2 kilobases 5' to the $C_{\gamma 2b}$ gene, and, on MEP203, from the J_4 DNA segment to a site 2.5 kilobases 5' to the C_{μ} gene. The remaining part of the 3.6-kilobase V- $C_{\gamma 2b}$ intron of the complete $\gamma 2b$ gene and the sequence that follows this part are entirely homologous to the germ-line $C_{\gamma 2b}$ gene clone. The restriction enzyme maps of the three DNA clones shown in Fig. 3B confirm the results of the electron microscopic studies. Thus, the cleavage sites present in the first 2.2-kilobase portion of the V- $C_{\gamma 2b}$ intron are also present at the cor-

Table 2 The two conserved blocks of sequences near the V-J or V-D-J joining sites

J DNA segments	Nanomers	Heptamers	Ref(s)
$J_{\kappa 1}$	GGTTTTTGT 23	CACTGTG	7, 13
$J_{\kappa 2}$	AGTTTTTGT 23	CAGTGTG	7, 13
$J_{\kappa 3}$	GGGTTTTGT 21	CACTGTA	7, 13
$J_{\kappa 4}$	GGTTTTTGT 24	CACTGTG	7, 13
$J_{\kappa 5}$	GGTTTTTGT 23	CACTGTG	7, 13
$J_{\lambda I}$	GGTTTTTGC 12	CACAGTG	6
J_{H1}	AGTTTTAGT 22	GACTGTG	*
J_{H2}	GGTTTTTGT 23	TAGTGTG	*
J_{H3}	ATTTATTGT 21	CACAATG	*
	23	CAATGTG	*
J_{H4}	GGTTTTTGT 22	TATTGTG	*
	31	TACTATG	*
Basic sequence	GGTTTTTGT	CACTGTG	
V DNA segments	Heptamers	Nanomers	Ref.
$V_{\kappa 21C}$	CACAGTG 11	ACAAAAACC	7
$V_{\kappa 21B}$	CACAGTG 12	ACAAAAACC	†
VK41	CACAGTG 12	ACATAAACC	8
VK2	CACAGTG 12	ACATAAACC	5
$V_{\lambda I}$	CACAATG 22	TCAAGAACA	6
$V_{\lambda II}$	CACAATG 23	ACAAGAACA	4
V_{H141}	CACAGTG 23	ACAAATACC	*
Basic sequence	CACAGTG	ACAAAAACC	

Two blocks of conserved sequences found in the 5'-flanking region of J DNA segments and in the 3'-flanking region of embryonic V DNA segments are compared. The numbers between the two types of sequences indicate the distance between them in base pairs. The bases different from those of the basic sequences in the corresponding positions are underlined. The refs from which the sequences were taken are listed (* this paper; † G. Heinrich, personal communication).

responding positions of the germ-line C_{μ} gene clone, whereas the cleavage sites located in the rest of the intron and the DNA stretch that follows are also found in the corresponding regions of the germ-line $C_{\gamma 2b}$ gene clone. The maps show that switch recombination occurs between a pair of sites, one located within the 750-base pair *HindIII*-*HindIII* segment of the germ-line C_{μ} gene clone and the other within the 200-base pair *EcoRI*-*BamHI* segment of the germ-line $C_{\gamma 2b}$ gene clone, and generates the 650-base pair *HindIII*-*BamHI* segment present in the complete $\gamma 2b$ gene clone (see Fig. 3B).

To understand this novel somatic event better, we determined the nucleotide sequences around the switch recombination sites (Fig. 5a). As expected, the 5' portion of the M141-P21 sequence is the same as the sequence of the germ-line C_{μ} gene clone, MEP203, whereas the 3' portion is identical to the sequence of the germ-line $C_{\gamma 2b}$ gene clone, MEP3. The exact μ - $\gamma 2b$ switch recombination sites are indicated by a vertical line in Fig. 5a. We propose to refer to the μ - $\gamma 2b$ switch sites on the germ-line C_{μ} gene and the germ-line $C_{\gamma 2b}$ gene as $S_{\mu}(\gamma 2b)$ and $S_{\gamma 2b}(\mu)$, respectively.

Sequences around the μ - $\gamma 2b$ switch sites

Are there any characteristic sequences around the μ - $\gamma 2b$ switch sites that might be considered as part of the recognition signal for the recombinase? As shown in Fig. 5a, two short blocks of sequences, TCCTGG and AGA, present in front of (towards the 5' side of) $S_{\mu}(\gamma 2b)$ are also present near the $S_{\gamma 2b}(\mu)$ site in the equivalent positions. The two sets of sequences are in the same orientation relative to the direction of transcription, and thus, differ from those sequences conserved near the V-J joining sites not only in the sequence content but also in the relative

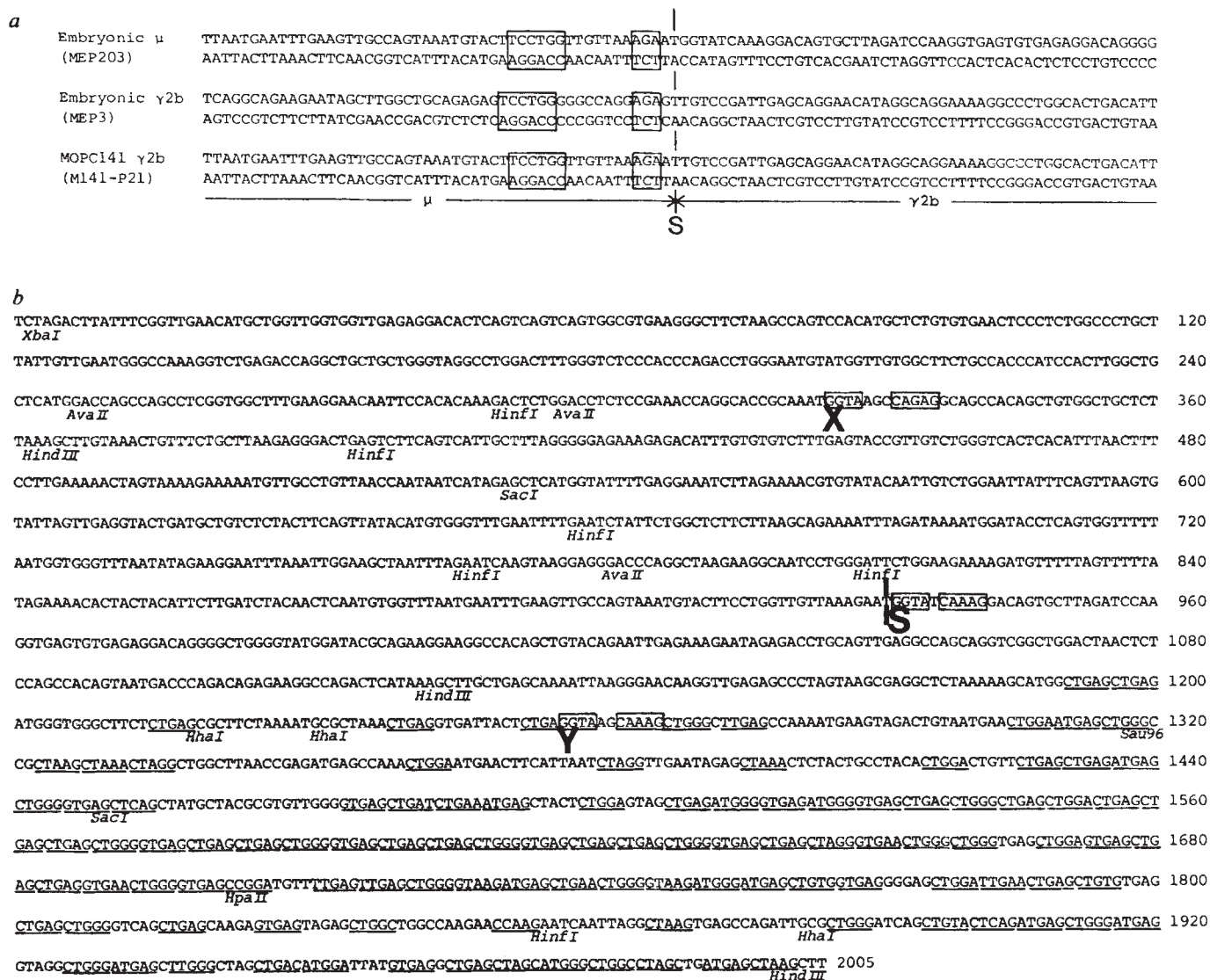


Fig. 5 *a*, Nucleotide sequences around the μ (γ 2b) switch recombination sites in clones MEP203, MEP3 and M141-P21. Sequencing strategy is shown in Fig. 3*B*. The recombination sites are indicated by the vertical line designated by S. The two blocks of sequences commonly present in the 5' side of the recombination sites of the embryonic μ and γ 2b gene clones are in boxes. *b*, The nucleotide sequence of 5'-flanking region of C μ DNA surrounding the switch recombination site for M141 γ 2b gene. The 1.8-kilobase *Xba*I-*Hind*III segment (Fig. 3*Bd*) was sequenced according to the strategy shown in Fig. 3*Bd*. Two regions (X and Y) homologous to the M141 γ 2b switch site (S) are indicated and common sequences GGTA and CAPuG are in boxes. Pentameric sequence CTGAG or its related sequences are underlined.

orientation. Although it is not clear whether these sequences are indeed recognized by the recombinase, the results suggest that the enzymatic mechanism for the μ - γ 2b switch recombination is quite different from that for V-J joining.

Sequences of the 5'-flanking region of the germ-line C μ gene

Recently, Davis *et al.*²⁰ isolated a complete α heavy-chain gene clone from MOPC603 that carries both V_H and C_α genes. Heteroduplex analysis of this clone against the germ-line C_α and C_μ gene clones suggested that the complete α heavy-chain gene is also generated by a switch recombination that involves the 5'-flanking region of the germ-line C_μ gene. The $S_\mu(\alpha)$ site was mapped between the two *Hind*III sites at 2.2 and 1.3 kilobases, respectively, from the C_μ gene (see Fig. 3*Bc*). Thus, the μ - α and μ - $\gamma 2b$ switch recombinations seem to use different sites in the 5'-flanking region of the germ-line C_μ gene. This suggests that

there may be several different switch sites in this region, possibly one at least for each of the six heavy-chain classes or subclasses, with the possible exception of the δ chain. (The behaviour of B cells suggests that μ - δ switches probably do not occur^{29,30}.)

We therefore extensively sequenced the 5'-noncoding region of the germ-line $C\mu$ gene clone, MEP203. Fig. 3*Bd* shows the restriction map of the 2.2-kilobase region preceding the $C\mu$ gene, and its complete nucleotide sequence is shown in Fig. 5*b*. We searched for sequences that had a resemblance to those found near the $S\mu(\gamma 2b)$ site and found two, which are referred to as X and Y and are located, respectively, around 0.6 kilobases upstream and 0.3 kilobases downstream of the $S\mu(\gamma 2b)$ site (Fig 5*b*). All three sequences share a tetranucleotide, GGTA, and a pentanucleotide, CAPuAG. However, the two sequences, TCCTGG and AGA, commonly present in the regions 5' to the $S\mu(\gamma 2b)$ and $S\gamma 2b(\mu)$ sites, are absent in the vicinities of X and Y. It is premature to predict that X and Y are indicative of switch recombination sites. Cloning and sequence analysis of complete heavy-chain genes of various classes and subclasses will clarify

the matter. (Recently, we learned that the Y sequence is indeed used for the recombination of the $\gamma 1$ gene sequenced by Kataoka *et al.*²¹ (N. Takahashi and T. Honjo, personal communication).)

In the 0.8-kilobase region extending from the 5' side of the Y sequence to the *Hind*III site, a pentameric sequence, CTGAG, or its close variants are repeated in tandem. As the $\Sigma\mu(\alpha)$ site reported by Davis *et al.*²⁰ falls within this region, involvement of the repeated sequence in the μ - α switch recombination can be invoked. Indeed, our recent DNA sequencing study on the germ-line C α gene clone indicates that the same pentameric sequences are prominent in the region where the C $\alpha(\mu)$ site had been mapped by Davis *et al.* (H.S., unpublished observations).

Clone MEP203 carries a deletion of about 3 kilobases which seems to have occurred during the cloning or subsequent propagation of the clone in *Escherichia coli*. Restriction enzyme maps of several independent C μ gene clones isolated by our laboratory and another laboratory²⁰ suggest that most clones carry deletions of various lengths that map between the two *Hind*III sites surrounding the Y sequence. As shown in Fig. 5b, this is the region where the pentameric sequences are repeated. Our recent DNA sequencing studies on two independent C μ gene clones carrying different deletions confirmed that these deletions occur within the pentamer-rich regions.

The two types of somatic recombination

The present studies deal with two types of somatic recombination required for the generation of complete heavy-chain genes, namely V-D-J or V-J joining and switch recombination. The two types of recombination are clearly different in several aspects, although they both occur somatically during lymphocyte differentiation. Although V-D-J or V-J joining should precede the synthesis of the free μ chain detected in pre-B cells³¹, switch recombination almost certainly occurs after these cells mature to small B cells bearing complete IgM molecules on the cell surface. V-D-J or V-J joining takes place at the margins of two protein-encoding DNA segments and generates complete V genes. In contrast, switch recombination occurs in the sequences located away from the protein-encoding DNA segments and in effect replaces the C μ exons of the complete μ -chain gene with exons coding for the C regions of other heavy-chain classes or sub-classes, except, perhaps, for the δ class.

In V-D-J or V-J joining, the recombinant DNAs are to be translated in the recombined regions. This imposes certain restrictions on the precise cutting and joining sites because the encoded polypeptide chains would have to fold properly to be able to function as immunoglobulin subunits. Indeed, this type

of recombination seems to occur within a short (several base pairs) region adjacent to the conserved palindromic heptamer present at the margin of every V and J DNA segment studied to date. However, the recombination site does not seem to be unique in a given gene segment and this flexibility, however limited, seems to have been exploited during evolution to increase the genetic capacity of the organism's antibody repertoire. Switch recombination can *a priori* occur anywhere within the 7.5-kilobase intron separating the J DNA segments and the C μ exons, and anywhere within a several-kilobase region located 5' to the respective C exon of other classes, as long as the transcription unit on the recombined DNA can encompass the V and C exons and the transcript can be processed properly to the mature mRNA. This suggests that switch recombination sites may be scattered widely in these regions. Indeed, the $\Sigma\mu(\gamma 2b)$ site for the MOPC141 $\gamma 2b$ chain and the $\Sigma\mu(\alpha)$ site for the MOPC603 α chain are at least 500 base pairs apart. Furthermore, our recent studies demonstrated that in the *Eco*RI digests of various myeloma DNA, the sizes of the DNA fragments detected by the 3-kilobase *Xba*I-*Xba*I fragment carrying the switch region (see Fig. 3Bc) are diverse, even among those myelomas secreting the heavy chains of the same class or subclass (R.M., unpublished observations). This suggests that one or both switch recombination sites are not class or subclass specific.

We previously proposed that the V-J joining can be considered as a reversal of an ancient, accidental insertion of an IS-like DNA element carrying invertedly repeated sequences at the margins⁷. In contrast, the evolution of immunoglobulin chains suggests that the C exons of various heavy-chain classes or subclasses arose by duplication of ancient C μ exons and their flanking sequences⁴⁴. The switch recombination sites have probably arisen from these duplicated flanking sequences by making use of the sequence homology among them. Whether the switch to various classes or subclasses is actively controlled at the level of the DNA recombinations remains to be determined.

Early *et al.*³³ have independently reported the analysis of M603 α heavy-chain V DNA and its germ-line V DNA and J DNA. They found that the 18-base pair D DNA segment in M603 V DNA is not accounted for by either germ-line V DNA or J DNA. They also identified two heavy-chain J DNAs by nucleotide sequencing that correspond to our J_{H1} and J_{H2}. Our determined or predicted joining sites on J_{H1} DNA for M603, M167 and S107 heavy-chain genes are in agreement with their direct sequencing of cDNAs for heavy-chain mRNAs prepared from respective myelomas.

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The cDNA for the β -subunit of human chorionic gonadotropin suggests evolution of a gene by readthrough into the 3'-untranslated region

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A 579-base pair approximately full-length cDNA which codes for the 145-amino acid long β -subunit of human chorionic gonadotropin (HCG) has been cloned in the plasmid vector pBR322 and its complete nucleotide sequence determined. A hydrophobic presequence of 20 amino acids can be identified from the nucleotide sequence. The amino acid sequence of the β -subunit is known to be related to those of the β -subunits of the other glycoprotein hormones LH, FSH and TSH, but the β -subunit of HCG is unique in that it contains a C-terminal extension of about 30 amino acids which has no homologous counterpart in the other three hormones. Analysis of the β HCG cDNA nucleotide sequence suggests that this extension may have arisen by the loss of the termination codon of an ancestral β -like gene so that most of what was previously the 3'-untranslated region now codes for protein. The β -subunit of HCG terminates with the codon UAA located 16 bases before the poly(A) in the sequence AAUAAA. This sequence is believed to be a recognition signal involved in either polyadenylation or processing and therefore has a dual role in this gene, serving both a coding and a regulatory function.

HUMAN chorionic gonadotropin (HCG) is a member of a group of four glycoprotein hormones which are functionally and structurally related. The other three hormones in the group, luteinizing hormone (LH), follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH), are synthesized in the anterior pituitary, whereas chorionic gonadotropin is synthesized in large amounts by the placenta. The level of HCG reaches a maximum about 10–12 weeks after conception and declines towards the end of pregnancy. The hormone functions to stimulate the corpus luteum to produce steroid hormones which are essential for the maintenance of pregnancy (for reviews see refs 1, 2). An antigen which is immunologically similar to HCG is also produced by many tumour tissues and transformed cell lines³ as well as by certain bacterial strains⁴.

All four hormones are glycosylated and consist of an α - and a β -subunit⁵. The same α -subunit is common to all four hormones and in the case of humans is 92 amino acids long^{6–11}, whereas the β -subunits have sequences unique to each hormone and confer biological specificity.

Comparison of the β -subunits reveals that they are all related. In the human the β -subunits of HCG, LH, FSH and TSH are 145, 115, 118 and 112 amino acids long, respectively^{7,12–16}. The β -subunit of HCG is thus about 30 amino acids longer than those of the other three hormones. The highest level of homology observed (82%) is between the β -subunits of HCG and LH, the homologies between all other pairs of β -subunits ranging from about 30 to 40%. Alignment of the β -subunit amino acid sequences shows that the extra 30 amino acids of the HCG β -subunit form a C-terminal extension that is unique to HCG and thus has no homologous counterpart in the other three β -subunits.⁷ The function of this extension is not known.

We have described previously the isolation by cloning in the plasmid vector pBR322 of a 621-base pair cDNA fragment, synthesized from first trimester placental mRNA, which codes for the entire α -subunit¹⁷. The results obtained when this cDNA fragment is used to hybridize to human genomic DNA digested with various restriction enzymes imply that the α -subunit is coded for by a single gene (ref. 17 and unpublished results).

The present report describes the isolation, also from first trimester placental mRNA, of a 579-base pair cDNA fragment which codes for the 145 amino acids of the β -subunit of HCG and for a previously unknown 20 amino acid-long presequence.

Examination of the sequence of the β HCG cDNA reveals that the C-terminal extension has probably arisen by the loss of a termination codon in an ancestral β -like gene so that the 3'-untranslated region now codes for amino acids. The sequence AAUAAA (ref. 18) in the β -subunit of HCG has the dual function of containing the termination codon (UAA) of the gene and a possible recognition site for polyadenylation or processing of the mRNA.

Cloning of the cDNA for the β -subunit of HCG

First trimester placental polyadenylated RNA has been shown previously^{17,19–22} by *in vitro* translation and immunoprecipitation of the products to be an enriched source of the mRNAs coding for both subunits of HCG. However, there seems to be an excess of α over β mRNA; this finding correlates with the excess of free α -subunit polypeptide over the β -subunit in the placenta²³.

The cDNA for the α -subunit of HCG was identified previously on the basis of restriction enzyme analysis. Total first trimester placental cDNA was digested with a variety of restriction endonucleases and the products were visualized by electrophoresis on 5% polyacrylamide gels. Prominent restriction enzyme fragments were observed and it was assumed that these arose by digestion of the major cDNA species, the α -subunit of HCG. Recombinant plasmids were therefore screened by restriction enzyme digestion of their DNA and those containing fragments which corresponded in size to those observed in total cDNA were considered potential α HCG recombinants. Sequence analysis confirmed the identity of one such recombinant.

This approach, however, has the disadvantage that there is no positive correlation between prominent restriction enzyme fragments and a particular mRNA species until DNA sequences have been determined. Potential recombinants for the β HCG cDNA were, in fact, isolated in this way but were found by sequencing not to code for the β -subunit.

A more direct approach is to use the known amino acid sequence of a protein to predict the existence of a restriction enzyme site in the gene. Most restriction enzymes do not have recognition sequences which are uniquely determined by certain

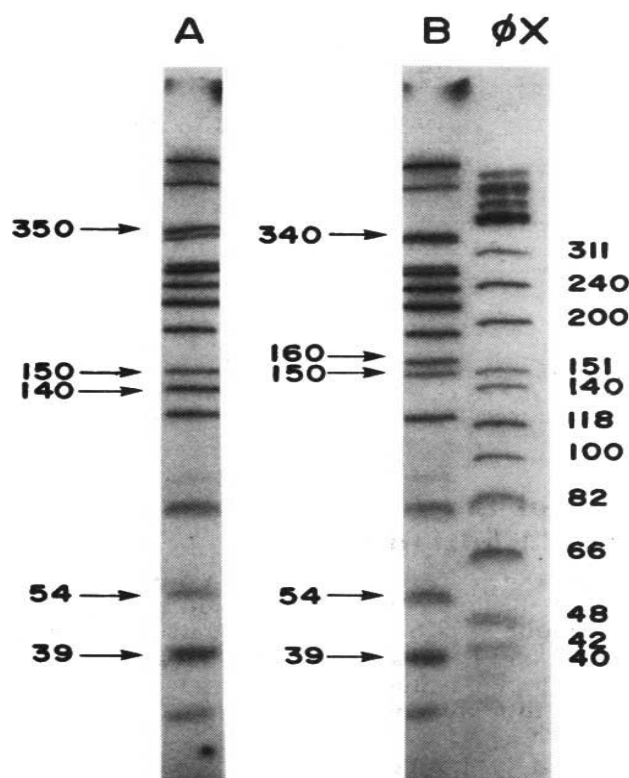


Fig. 1 Restriction enzyme analysis with *Sau96I* of the two recombinants between pBR322 and the cDNA of the β -subunit of HCG. The recombinants were obtained from first trimester placental cDNA in the size range of 500–700 base pairs using the approach of adding synthetic *HindIII* oligonucleotide linkers to the cDNA. Experimental details were as described previously¹⁷ with the exception that strain RRI was used in place of χ 1776. All experiments conformed with the NIH Guidelines for Recombinant DNA Research. Overnight cultures in L broth (3 ml each) were grown for 72 recombinants and the cells were pelleted. Cleared lysates were made using lysozyme, EDTA and Triton⁴⁸ and were dialysed extensively against 0.3 M NaCl, 10 mM Tris-Cl, pH 8.0, and 1 mM EDTA before precipitation with ethanol. Each plasmid DNA preparation was then resuspended in 10 μ l H₂O and 1 μ l of the DNA was digested at 37 °C for 30 min with 1 unit of *Sau96I* (Biolabs) in a 5 μ l reaction containing 50 mM NaCl, 6 mM Tris-Cl, pH 7.5, 6 mM MgCl₂ and 1 mM dithiothreitol. The sample was then used directly to resuspend 1 μ Ci of [α -³²P]dGTP which had been evaporated to dryness and 0.5 units of DNA polymerase I (Klenow modification) were added before incubation at room temperature for 15 min (ref. 26). The sample was then electrophoresed directly on a 0.3-mm thick 5% polyacrylamide gel until the bromophenol blue marker dye was about 5 cm from the bottom of the gel. The gel was then soaked for 15 min in 10% acetic acid to elute free [α -³²P]dGTP and was exposed overnight to X-omat R film at -70 °C with an intensifying screen. Lanes A and B show autoradiographs of the *Sau96I* digests of the two pBR322- β HCG recombinants. The sizes of the fragments corresponding to the β HCG cDNA are indicated (see text). The third lane is a *HindIII* digest of Φ X174 RF DNA used as size markers.

amino acid pairs, but two enzymes, *AsuI* (ref. 24) and *Sau96I* (ref. 25), are particularly useful because they recognize the sequence GGNCC. As glycine is coded for by the four codons GGN and proline by the four codons CCN, every Gly-Pro dipeptide must be represented by an *Sau96I* site in the corresponding gene. Other groups of amino acids may also be coded for by sequences containing *Sau96I* sites, as the sequence GGNCC may be translated in two other reading frames.

As there are Gly-Pro dipeptides at positions 102 and 103 and positions 136 and 137 in the β -subunit of HCG^{7,12,13}, the corresponding gene must have *Sau96I* sites 102 base pairs apart. Within this 102 base pairs of DNA, at amino acid positions 104–106 and 122–124, are the sequences Lys-Asp-His and Lys-Ala-Pro, respectively, which, with the appropriate codon choice, would also correspond to *Sau96I* sites. These restriction enzyme sites would result in the 102-base pair fragment being divided into pieces 9, 54 and 39 base pairs long.

Double-stranded cDNA was synthesized from first trimester placental RNA as described before (ref. 17 and Fig. 1 legend) and fragments in the size range of 500–700 base pairs were recovered from a preparative 5% polyacrylamide gel and cloned into the unique *HindIII* site of pBR322. Small-scale DNA preparations were made from 72 such recombinants and the DNAs digested with *Sau96I*. The resulting DNA fragments from the recombinant plasmids were labelled at their termini with [α -³²P]dGTP using DNA polymerase I (ref. 26 and Fig. 1) and analysed on 5% polyacrylamide gels.

Two of the recombinants were observed to have *Sau96I* fragments 54 and 39 base pairs long (Fig. 1) and were thus considered potential β HCG clones. The 9-base pair fragment would not be observed on a 5% polyacrylamide gel. In addition to these fragments, a 150-base pair fragment is common to both recombinants. A fragment of this size is compatible with the amino acid sequence for the β -subunit of HCG, as the sequence Pro-Ala-Leu at positions 50–52 could be coded for by a nucleotide sequence containing *Sau96I* site, which is about 150 bases from the *Sau96I* site corresponding to the Gly-Pro dipeptide at positions 102–103.

The other *Sau96I* fragments, 350 and 140 base pairs long in the case of A and 340 and 160 in the case of B, were shown by combined *HindIII* and *Sau96I* digestion (data not shown) to be terminal fragments containing pBR322 sequences in addition to cloned sequences. The two recombinants were thus considered potential candidates for containing β HCG cDNA sequences and seem to represent slightly different lengths of copying of the same mRNA species.

DNA sequence analysis of the β HCG cDNA

Both potential β HCG recombinants were positively identified by DNA sequence analysis. The plasmid DNAs were digested with *HindIII*, labelled with polynucleotide kinase and [γ -³²P]ATP, and the 5'-terminal *HindIII*-*Sau96I* fragments sequenced. Subsequently, the complete sequence of recombinant A, which is about 10–20 nucleotides longer than B at the 5' end, was determined. Both the chemical method of Maxam and Gilbert²⁷ and the chain-termination method of Maat and Smith²⁸ were used, because both methods used the same 5'-terminally labelled double-stranded DNA fragments. The sequence was determined on both strands of the DNA to ensure accuracy.

The complete sequence of recombinant A, designated pBR322-c β HCG, is presented in Fig. 2. The cDNA is 579-base pairs long, of which 40 represent the poly(A) sequence. All 145 codons which correspond exactly to known amino acid sequence of β HCG can be identified^{7,12,13}.

A hydrophobic presequence of 18–29 amino acids, which is processed to generate the mature polypeptide, is observed in secretory proteins (see ref. 29 for a compilation of presequences). The α -subunit of HCG has a 24 amino acid-long presequence^{17,30}, but no information is available on the presequence of the β -subunit. If the sequence preceding the serine codon at position 1 in β HCG is read in triplets towards the 5' end, potential ATG initiation codons are observed at positions -18 and -20 (see Fig. 2). No other ATG codons are observed between this position and the 5' end of the cloned cDNA in this or the other two reading frames. It has been proposed, on the basis of examining the 5'-untranslated sequences of 22 eukaryotic mRNA species, that the initiator methionine codon is the AUG located closest to the 5' end of the mRNA³¹. Two exceptions to this rule have been observed with the mRNAs for the VP1 in the virus SV40^{32,33} and for bovine preproparathyroid hormone³⁴, but in both cases it has been proposed that the AUG triplet in the 5'-untranslated region is masked by secondary structure. The β -subunit of HCG can thus be predicted to have a presequence 20 amino acids long, but the existence of a larger presequence (greater than 28 amino acids long) cannot be definitely ruled out because there are no

termination codons in phase in the part of the 5' untranslated region which has been cloned. Characteristically, the presequence of β HCG is hydrophobic, with the somewhat unusual structure of seven consecutive leucine residues at positions -8 to -14.

The nucleotide sequence of the β HCG cDNA has a very high G + C content (G 28.0%, C 38.0%, A 16.3%, T 17.6%) when compared with total human DNA (G 19.5%, C 19.8%, A 30.9%, T 29.6%). A similar bias towards a high G + C content is also observed in the human cDNAs for insulin³⁵ and chorionic somatomammotropin (ref. 36 and E. Tischer, J.C.F. and H.M.G., unpublished data), but is not so noticeable in growth hormone³⁷, the α -subunit of HCG¹⁷ or β -globin³⁸⁻⁴⁰.

Consistent with the high G + C content of β HCG cDNA is the very considerable bias towards codons ending in G or C (G 31.9%, C 50.6%, A 7.2%, T 10.2%). A compilation of the nucleotides present in the third codon position of the known human cDNA sequences is given in Fig. 3. The high incidence of codons ending in G and C seems to be a common feature of human genes even with growth hormone, β -globin and α HCG, which do not have a marked overall high level of G and C. These data are consistent with the observation⁴¹ that codon choice is species specific and, if this has a role in modulating translation, may present difficulties in obtaining high levels of expression of human genes in other cell types.

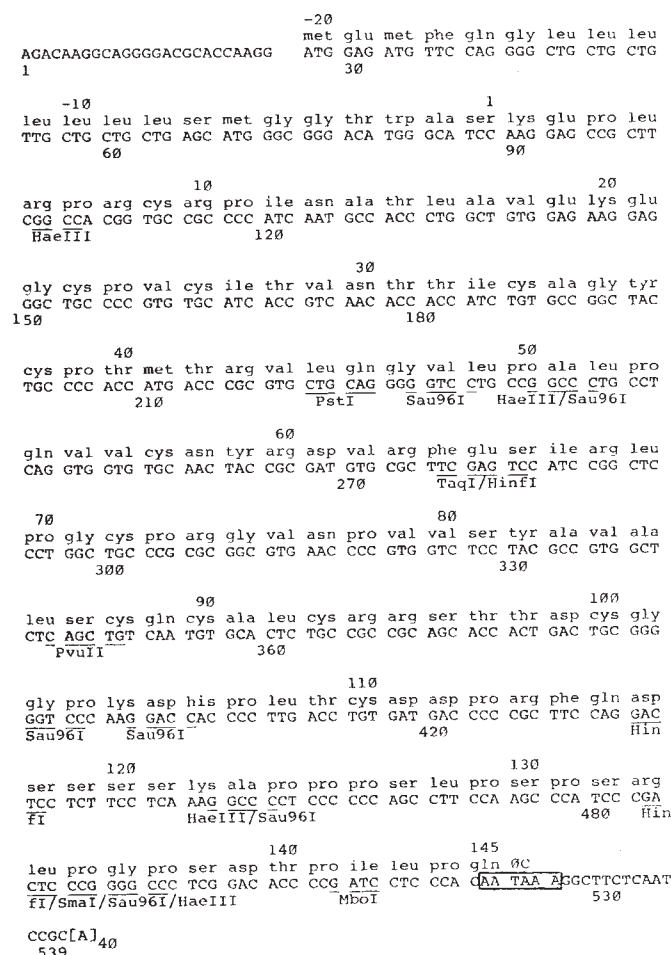


Fig. 2 Nucleotide sequence of the 579-base pair cDNA fragment coding for the β -subunit of HCG. The amino acid sequence of the 145-amino acid long protein^{7,12,13} is shown as well as the 20-amino acid long presequence deduced from the nucleotide sequence. Nucleotides are numbered below the sequence and amino acids above the sequence. Only those restriction enzyme sites used in the sequence analysis are indicated. The AAUAAA sequence at the 3' end of the coding region is marked.

cDNA	Base			
	A	T	G	C
h α CG	25	28	29	35
h β CG	12	17	53	84
hGH	31	29	65	93
hCS	17	17	60	75
hInsulin	14	8	43	45
h β Globin	10	40	53	45

Fig. 3 Frequency of third position codon choices in the known human cDNA sequences; β HCG, α HCG¹⁷, growth hormone (GH)³⁷, chorionic somatomammotropin (HCS)³⁶, insulin³⁵ and β -globin³⁷⁻³⁹.

Evolution of the genes for the glycoprotein hormones

The β -subunits of the four glycoprotein hormones HCG, LH, FSH and TSH all show amino acid sequence homologies which range from about 30% between HCG and FSH to about 80% between HCG and LH. Alignment of all four sequences shows that the 12 cysteine residues are completely conserved and that there is an additional conserved region located at positions 34-38 in β HCG.

A much less pronounced homology between the α - and β -subunits of bovine TSH has also been proposed by Pierce⁴², leading to the suggestion that all glycoprotein hormones genes evolved from a common ancestor which subsequently diverged to generate α and β sequences. This amino acid sequence homology can also be detected between α and β HCG and is presented in Fig. 4. The alignment used here differs somewhat in the N-terminal region from that proposed from bovine TSH and can now also be extended to include the presequences. Overall, the homology is very low (16% not including the deletions and insertions), but there is still a very noticeable conservation of the cysteine residues, with 6 of the 12 being maintained between the α and β -subunits.

Figure 4 also shows the alignment of the coding nucleotide sequences for the α - and β -subunits of HCG based on the same alignment as used with the amino acid sequences. Overall, the homology level is still very low (31%), but there are certain areas which are considerably more closely related, especially in the regions of the cysteine residues. The observed similarity between the sequences does not merely reflect a similar bias in base composition between the two species, for the base composition of the α cDNA (G 20.0%, C 24.5%, A 26.6%, T 29.0%) is very different from that of the β cDNA (G 28.0%, C 38.0%, A 16.3%, T 17.6%).

The nucleotide sequence data therefore tend to confirm the proposal that the α and β genes are related and thus probably have evolved from a common ancestor, as the homology previously detected at the amino acid level is somewhat more apparent when the nucleotide sequences are considered. In this respect, therefore, the glycoprotein hormone genes may be similar to the globin genes, which are also assumed to have evolved from a common precursor⁴³ into an α gene and a family of several β -like genes.

Readthrough into the 3'-untranslated region

The 3'-untranslated region of the β HCG mRNA is only 16 bases long and is thus remarkably short when compared with other eukaryotic mRNA species (see Fig. 2). The termination codon UAA is located in the sequence AAUAAA, which is found in the 3'-untranslated regions of all polyadenylated eukaryotic cellular mRNAs¹⁸ at about 15-20 bases from the poly(A). The lack of this sequence in some non-polyadenylated plant viral mRNAs⁴⁴ led to the proposal that the AAUAAA is

involved in the process of polyadenylation⁴⁵. However, there is an exception to this, as the genomic RNAs of several picornaviruses are polyadenylated but do not have the AAUAAA sequence⁴⁶. Thus, it may be that in the case of the cellular mRNAs this sequence acts as a signal in the processing of a larger precursor mRNA molecule as well as being involved in polyadenylation. In β HCG mRNA, this sequence therefore has a dual overlapping function, as it codes for the last codon, CAA (Gln), and for the terminator (UAA), in addition to the proposed regulatory function.

The alignment of the β -subunit amino acid sequences shows that the β -subunit of HCG has a C-terminal extension of about 30 amino acids which has no homologous counterpart in the other three. As the β HCG mRNA has such an unusually short 3'-untranslated region, it can be proposed that the β -subunit of HCG evolved from a common β -subunit ancestor by some readthrough event so that the 3'-untranslated sequence became a coding region. This could have arisen by changes such as point or frameshift mutations or by an alteration of the splicing pattern of the precursor mRNA. Verification of this proposal will require sequence analysis of the genes for the other three β -subunits so that homologies between their 3'-untranslated regions and the C-terminal extension of β HCG can be investigated.

A similar observation has also been made with the abnormal human α -haemoglobin Constant Spring, which has a 31-amino

acid C-terminal extension when compared with normal α -globin⁴⁷. Sequence analysis of normal human α -globin cDNA showed that the carboxy-terminal amino acids of α -globin Constant Spring could be coded for by the 3'-untranslated region with a termination codon located in the same position as in β HCG³⁹.

The availability of cloned cDNA sequences for both subunits of HCG should facilitate further investigation of the structure and regulation of the gonadotropin genes. Hybridization probes can be generated to investigate the cellular genes to determine whether any linkage exists between the related genes and to examine the synthesis of HCG in the placenta, transformed cells and, possibly, bacteria.

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Fig. 4 Alignment of the amino acid and nucleotide sequences of the coding regions of the α (lower lines) and β (upper lines) subunits of HCG. To maximize the homologies, one insertion of five codons, and two deletions of one and six codons have been proposed in the α sequence. The overall sequence homologies are 16.1% and 31.0% for the amino acids and nucleotides, respectively. These values do not take into account the deletions and insertions. Only the region of the β HCG coding sequence which has a homologous counterpart in the α -subunit (amino acids -20 to 99) is shown.

LETTERS

No IR burst from the X-ray rapid burster MXB1730-335

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The X-ray rapid burster MXB1730-335, discovered by Lewin *et al.*¹ has been identified with a compact globular cluster embedded in the midst of the galactic plane and named² Liller 1. It has an extremely large interstellar extinction ($A_v = 11$ mag) from which Liller² and Kleinmann *et al.*⁴ derived its distance as 10 kpc. Kulkarni *et al.*³ detected IR bursts in the globular cluster, six times during their 2.5 h observing time on 4-5 April 1979 using a 1-m telescope at Kavalur³. The burst power emitted in *H*-band was estimated to be $1.6-2.3 \times 10^{30}$ W. Stimulated by this report, we monitored MXB1730-335 using a multi-band IR photometer for about 10 h during April and August 1979. In the latter period, although the X-ray bursts were very active, no coincident IR and X-ray burst could be detected. We show here that this conflicts with previous results by Kulkarni *et al.*³ and Jones *et al.*⁵.

The source was observed on 20-22 April 1979 at *H*-, *K*- and *L*-bands using a multi-colour photometer attached to the 1-m IR telescope at the Agematsu IR Observatory of Kyoto University. During the monitoring time, ~4 h in three days, we could not detect any change of flux exceeding the statistical fluctuations; 2.7×10^{-17} W cm⁻² μm⁻¹ at the *H*-band, 1.8×10^{-17} W cm⁻² μm⁻¹ at the *K*-band and 3×10^{-17} W cm⁻² μm⁻¹ at the *L*-band.

The X-ray satellite HAKUCHO, launched in February 1979, registered a revival of X-ray burst activity from early August 1979. We again attempted to monitor this in the near-IR using the 61-cm telescope of the University of Hawaii on Mauna Kea where we were conducting a deep IR survey in the galactic plane. The observations were performed with a multi-colour photometer built for the survey. The photometer can monitor four colours: *I*(0.82 μm), *H*(1.61 μm), *K*(2.21 μm) and *L*(3.61 μm) bands simultaneously, using beam splitters in conjunction with a Si-photodiode (*I*-band) and three independent InSb photovoltaic detectors (*H*-, *K*- and *L*-bands). The time constant of the lock-in-amplifier was set at 0.3 s. The beam was

Table 2 Upper limits to the IR flux changes in association with the X-ray burst event at 12 August 06.38.27 (UT)

Band	Upper limits	
	Observed (W cm ⁻² μm ⁻¹)	Corrected for interstellar extinction (W cm ⁻² μm ⁻¹)
<i>I</i> (0.82 μm)	1×10^{-17}	1×10^{-15}
<i>H</i> (1.61 μm)	1×10^{-17}	6×10^{-17}
<i>K</i> (2.21 μm)	4×10^{-18}	1×10^{-17}
<i>L</i> (3.61 μm)	2×10^{-17}	3×10^{-17}

wobbled in N-S direction with a beam throw of 1.5 arc min at ~10 Hz.

No bursts were observed within the statistical fluctuation of the signals in any channel during the observing time of 6 h during 9, 10 and 12 August 1979. The details of the observations in April and August are summarized in Table 1; γ Sgr was chosen as a calibration star.

During the period in August, HAKUCHO was observing X-ray bursts almost every 20 or 30 min (ref. 6). From statistical considerations, therefore, it is improbable that an X-ray burst did not occur during our IR observations which lasted 6 h. However, the observations of HAKUCHO and that of the IR monitoring missed each other most of the time. Only in the following event was an X-ray burst accompanied by an IR observation.

An X-ray burst associated with IR bursts:

Onset time:	06.38.27 UT 12 August 1979
Burst duration:	126 s
X-ray burst flux:	1.3×10^{-15} W cm ⁻² (3-10 keV)

(Observation by HAKUCHO⁶)

As the X-ray observation was interrupted, its end time was not observed but this is undoubtedly one of the typical X-ray bursts of the so-called 'trapezoidal' form⁶. Figure 1 shows the IR raw data in *H*- and *K*-bands recorded on strip charts in the corresponding time of the X-ray burst event. The X-ray burst profile is also shown for comparison⁶.

We can find no change in the IR flux which is larger than statistical fluctuations before or after the onset of the X-ray burst. The r.m.s. fluctuations of the signals are 7×10^{-17} W cm⁻² μm⁻¹ and 2×10^{-17} W cm⁻² μm⁻¹ for *H*- and *K*-bands, respectively. Slight depressions labelled 'adj' were accidentally caused by the pointing adjustment of the telescope.

To improve the statistics, we integrated the observed fluxes of the source in its steady state before the onset of the burst and during on-burst time, from which upper limits for changes of the flux level can be estimated; the results are given in Table 2

Table 1 Journal of observations

Date (1979)	Time (UT)	Duration (min)	Bands	Beam (arc s)	Observatory
20 April	19.41-20.20	20	<i>K</i>	40φ	Agematsu
21 April	17.47-20.10	136	<i>H, K, L</i>	60φ	Agematsu
22 April	18.30-20.10	86	<i>H, K, H</i>	60φ	Agematsu
		Total: 242 = 4 h 02 min			
		Photometry: <i>H</i> (6.6 ^{mag}) <i>K</i> (5.8 ^{mag}) <i>L</i> (6.2 ^{mag})	Beam size: 50 arc s ² φ		
9 August	09.49-10.49	55	<i>I, H, K, L</i>	60 × 20	Mauna Kea
11 August	06.12-10.31	90	<i>I, H, K, L</i>	60 × 20	Mauna Kea
12 August	06.14-10.00	226	<i>I, H, K, L</i>	60 × 20	Mauna Kea
		Total: 372 = 6 h 12 min			
		Photometry: <i>I</i> (9.5 ^{mag}), <i>H</i> (6.2 ^{mag}), <i>K</i> (5.4 ^{mag}), <i>L</i> (5.6 ^{mag})	Beam size: 60 × 20 arc s ²		

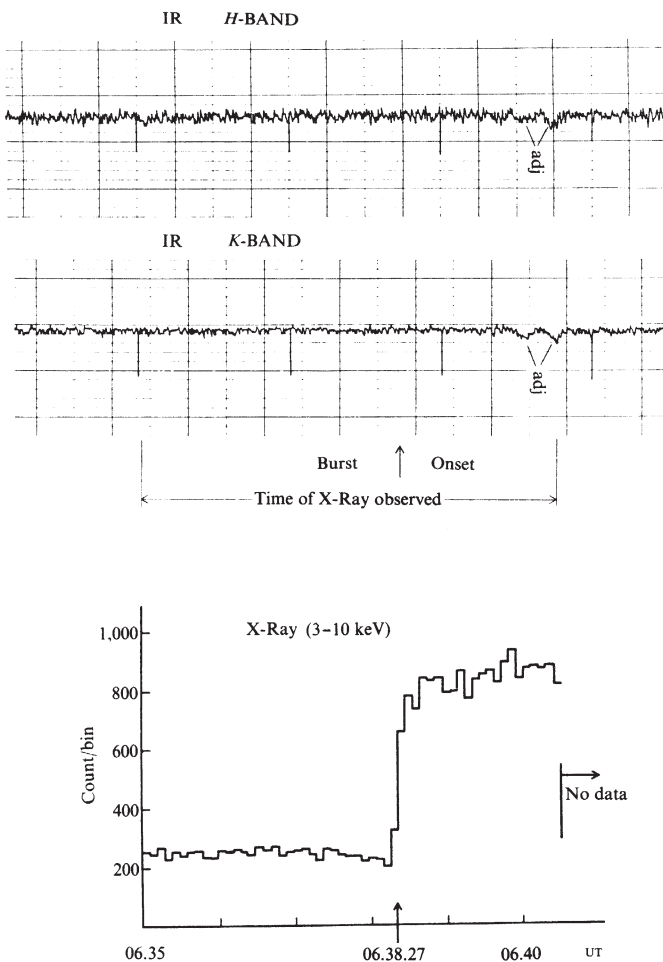


Fig. 1 IR observational data at *H*- and *K*-bands in correspondence with the X-ray burst event on 12 August 1979.

corrected for the interstellar extinction assuming $A_K = 1.1$ mag (ref. 4).

The intrinsic fluxes thus obtained for *K*- and *L*-bands are substantially smaller than the X-ray flux of $1.3 \times 10^{-15} \text{ W cm}^{-2}$ (3–10 keV) of the order of 10^{-2} . Assuming a distance of 10 kpc for the source, we estimate the upper limit to the energy release in the IR region to be of the order of 10^{29} W in a band width of $1 \mu\text{m}$. This is more than one order of magnitude smaller than that detected by Kulkarni *et al.*³. Recently, Jones *et al.*⁵ have announced another detection of IR bursts in the globular cluster on 5 September 1979, about a month later than our observations. Its energy release in *K*-band was estimated to be $2.2 \times 10^{30} \text{ W}$, compatible with the previous results obtained by Kulkarni *et al.*³.

The positive detections by Kulkarni *et al.*³ and by Jones *et al.*⁵ apparently disagree with our negative results. This may be attributable to difference in Types of the X-ray bursts. In fact Kulkarni *et al.* assumed their IR bursts to be associated with Type 1 X-ray bursts, while the X-ray bursts observed by the HAKUCHO were exclusively Type II bursts⁶.

The IR bursts so far observed are not accompanied by X-ray data. We need to make simultaneous observations in the X-ray and IR regions for confirmation of their exact concurrence, or their relationship to the burst Type. If they occur simultaneously, the emission should be of nonthermal origin; which means that magnetic field would be strongly coupled with the emission mechanism. Polarimetric observations would give data about such a mechanism and about the energy spectrum.

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The IR variability of SS433

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We report here the results of a one-year monitoring programme of SS433 at IR and visual wavelengths. The most striking feature of these observations is the near constancy of the IR colours while the source's flux varies over a range of ~ 1.2 mag. There is no evidence for the 13.1-day period first reported in radial-velocity measurements by Crampton *et al.*¹. However, it seems possible that an 11.8-day period is present.

SS433 remains an object of great interest because of the presence of enormously Doppler-shifted emission lines swinging back and forth across its spectrum^{3–5}. (Suggested models are given in refs 6, 7 and comprehensive reviews of the spectroscopic data in refs 8, 9.) The work reported here is not directly connected with the emission-line phenomena but provides

Table 1 JHK Photometry of SS433

JD	$J \pm \Delta J$	$H \pm \Delta H$	$K \pm \Delta K$
3942.369	9.73 0.04	9.10 0.02	8.45 0.02
3944.392		8.79 0.04	8.12 0.02
3945.367	9.41 0.04	8.75 0.02	8.12 0.02
3973.351	9.38 0.02	8.62 0.03	8.05 0.02
3978.359	9.26 0.02	8.51 0.03	7.94 0.02
4007.674	9.59 0.10	8.69 0.03	8.01 0.04
4012.676	9.35 0.02	8.65 0.03	7.99 0.02
4014.660	9.18 0.02	8.46 0.02	7.91 0.01
4015.640	9.25 0.02		
4019.080	9.57 0.05	8.77 0.05	8.17 0.05
4021.030	9.00 0.05	8.27 0.05	7.72 0.05
4023.070	8.79 0.05	8.01 0.05	7.47 0.05
4023.140	9.08 0.07	8.45 0.07	7.73 0.07
4024.010	9.35 0.07	8.68 0.07	7.88 0.07
4024.030	9.34 0.05	8.78 0.05	7.78 0.05
4025.080	8.95 0.07	8.20 0.07	7.28 0.07
4096.375	8.64 0.03	7.84 0.02	7.23 0.03
4097.458	9.23 0.04	8.51 0.04	7.76 0.04
4098.396	9.51 0.03	8.79 0.04	8.16 0.04
4099.375	9.76 0.03	9.11 0.02	8.32 0.03
4100.542	9.68 0.02	8.99 0.02	8.35 0.02
4101.563	9.54 0.02	8.74 0.02	8.13 0.02
4104.375	8.98 0.02	8.17 0.02	7.45 0.02
4107.500	9.04 0.02	8.22 0.02	7.41 0.02
4108.479	8.95 0.02	8.11 0.02	7.41 0.02
4109.458	9.12 0.02	8.32 0.02	7.67 0.02
4149.417	9.49 0.02	8.66 0.01	7.99 0.02
4150.375	9.20 0.01	8.43 0.01	7.73 0.02
4151.438	9.05 0.01	8.24 0.01	7.63 0.01
4152.344	8.76 0.02	7.93 0.01	7.26 0.01
4153.396	8.75 0.03	7.82 0.02	7.20 0.01
4157.396	9.43 0.04	8.50 0.03	7.96 0.04
4188.736	9.92 0.01	8.92 0.01	8.19 0.01
4189.713	9.28 0.01	8.48 0.01	7.84 0.01
4296.350	9.26 0.04	8.54 0.02	7.95 0.02
4354.258	9.09 0.01	8.48 0.01	7.86 0.01
4354.383	9.08 0.01	8.41 0.01	7.80 0.01
4355.385	9.17 0.01	8.49 0.01	7.89 0.01

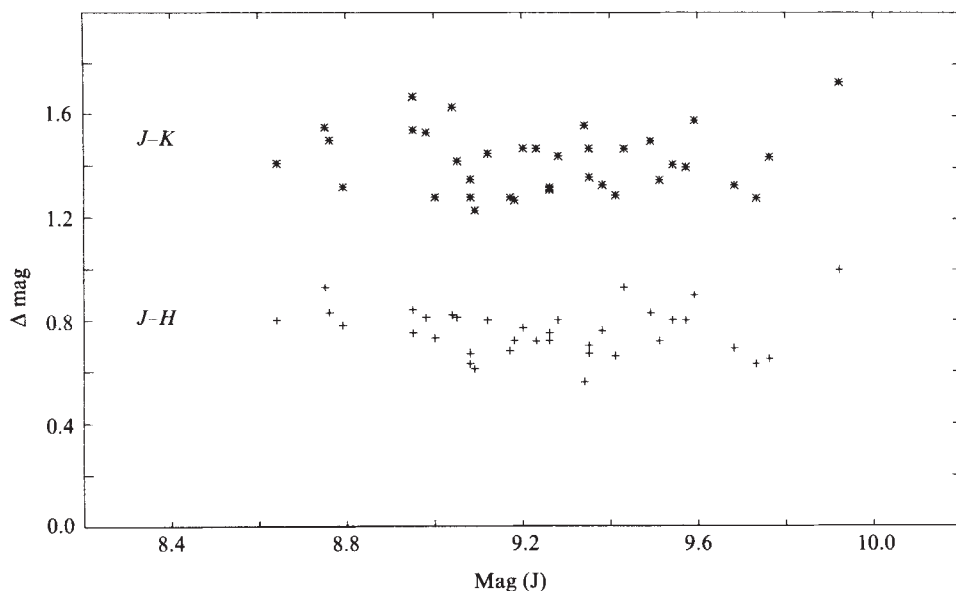


Fig. 1 $J-K$ (*) and $J-H$ (+) colours plotted against J magnitude for SS433.

additional information about the nature of the underlying binary system. The variability of the source has been studied at several wavelengths^{2,10-14} and the radial velocity period of 13.1 days first reported by Crampton *et al.*¹ has now also been detected in the $H\alpha$ line-to-continuum ratios¹⁵. A spectroscopic modulation of 13 days has also been reported by Liller *et al.*¹⁶.

We have observed SS433 between April 1979 and May 1980 with various instruments, mostly from the Cabezón Observatory, Tenerife, using the 1.5-m IR flux collector. At Tenerife, most of the data were obtained at $VJHK$ with the Leicester photometer or Hatfield polarimeter/photometer, which use the same InSb Dewar and photon counting systems. Additional V points were obtained at Tenerife with the RGO travelling optical polarimeter/photometer. Further JHK data were obtained at the AAT in Australia and at Ukirt in Hawaii. This collection of data has been supplemented with other published data^{17,18}.

All these data are presented in Tables 1-3 but here we mainly confine our discussion to the more abundant data in Table 1. The JHK measurements have been combined to produce Fig. 1 which shows the remarkable constancy of the IR colours. This is just as expected for free-free emission¹⁸⁻²⁰.

The J data in Table 1 have been folded at trial periods in the range 0.1-50 days using the technique of Bopp *et al.*²¹ to find the most likely period. This method is appropriate to a small sample of data containing large and irregular gaps in temporal coverage.

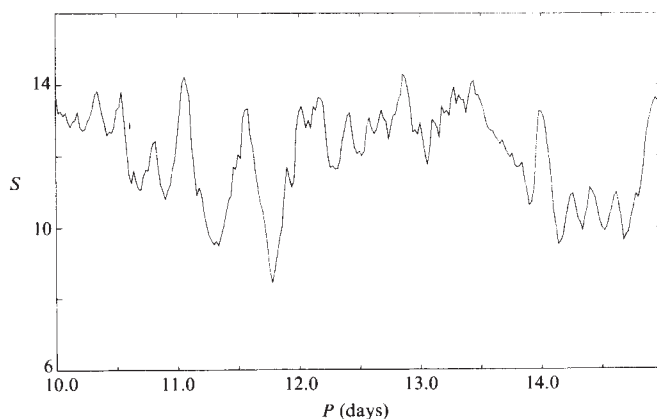


Fig. 2 An analysis of the J data for periodicities (Bopp *et al.*²¹). Sharp dips indicate periods of interest and the vertical scale is logarithmic ($S = \Sigma\Delta M$).

The results are shown in Fig. 2, and there is no indication of a 13.1-day period. This may be confirmed by folding the data at this period; no reasonable light curve results.

Originally Giles²² reported smooth JHK light curves for a period of 11.8 ± 0.05 days, possessing two minima, the broader one being used to define the ephemeris

$$1979 \text{ August } 14.26(\pm 0.5) \text{ UT} + 11.8E$$

This ephemeris was used to predict the occurrence of the possible narrow 'eclipse' feature²² which was then apparently

Table 2 V Photometry of SS433

JD	V	$\pm\Delta V$
4007.674	13.64	0.08
4012.676	13.86	0.03
4014.660	13.59	0.02
4101.563	14.24	0.02
4103.375	13.67	0.02
4107.500	13.55	0.02
4108.479	13.86	0.02
4109.458	13.95	0.02
4131.396	14.00	0.02
4132.382	13.98	0.04
4133.344	13.95	0.03
4134.354	14.01	0.02
4139.350	13.78	0.03
4140.350	13.71	0.03
4141.351	14.00	0.02
4142.344	14.12	0.02
4143.354	14.13	0.02
4149.417	14.60	0.10
4150.375	14.46	0.08
4151.438	14.37	0.07
4152.344	14.04	0.05

Table 3 B, I Photometry of SS433

JD	B	$\pm\Delta B$
4012.676	15.42	0.06
4014.660	15.20	0.04
4015.640	15.27	0.04
4150.375	16.18	0.22
4151.438	16.03	0.18
4152.344	15.70	0.25

JD	I	$\pm\Delta I$
4096.375	9.26	0.30
4097.458	10.16	0.20
4098.396	10.76	0.40

confirmed by several subsequent observations at UKIRT in Hawaii. Further points obtained at the AAT in early 1980 also apparently confirm this feature, in phase although the width and depth are undefined and probably variable. The dip around 11.8 days from the Bopp analysis of Fig. 2 naturally produces a reasonable light curve when the data are folded at this period. Figure 3 shows the resulting J light curve. The apparent smoothness of this curve is somewhat less than that evident for the first attempt²² now that more points have been added. SS433 may well have some variability over long time scales in addition to that caused by any binary period modulation.

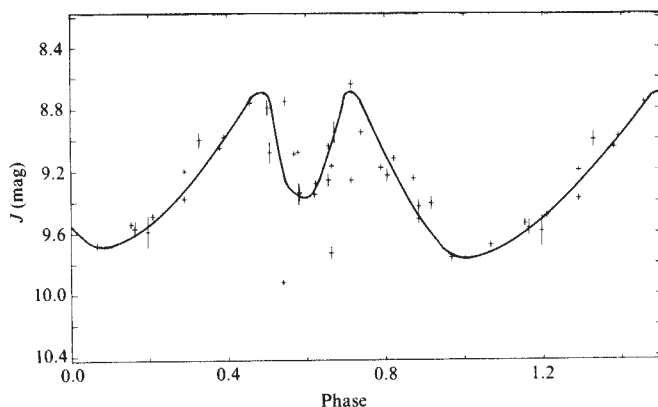


Fig. 3 J light curves for the data folded at 11.8 days.

We note that the photometry by Glass (personal communication) agrees with our range of intensity and colour variation but is not entirely consistent with our possible period. Such differences may prove important when a much larger data base is available if they can be related to the 164-day period. It is clearly important that the existence or otherwise of this 11.8-day period should be demonstrated over the coming summer. This will best be achieved by ~ 3 weeks of complete coverage rather than by folding irregularly spaced short data sets. The limited optical data we have obtained shows no periodicity so we are unable to comment on the 6.45/12.9-day period reported by Kemp *et al.*²³ or the 12.8-day period of Gladyshev *et al.*²⁴.

Given the unprecedented nature of SS433 it does not seem inconceivable that the source should have two periods, only one or even neither of which is a true orbital period. SS433 will clearly repay further detailed photometric study. Observations of any modulation in the X-ray flux or low-energy cutoff would be of special interest.

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Ultrasonic imaging in scanning electron microscopy

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A new type of microscopy is described here which uses a focused, modulated electron beam to generate ultrasonic waves at the front surface of a specimen and a piezoelectric transducer to detect these waves at the rear surface. The transducer output is used to form a scanned, magnified image of the specimen. A unique feature of this technique is that image contrast comes primarily from spatial variations in thermal and elastic properties. Images of integrated circuits have been obtained with $\sim 4 \mu\text{m}$ resolution. In thin film specimens $0.1 \mu\text{m}$ resolution should be possible.

This mode of ultrasonic imaging in scanning electron microscopy is called electron-acoustic microscopy in analogy with photoacoustic microscopy¹⁻⁵ in which a laser is similarly used to generate acoustic or ultrasonic waves by thermoelastic expansion. An advantage of electron-acoustic microscopy is that the electron beam can be focused to a much smaller spot than is possible with visible light; and with proper attention to problems of electron beam spreading within the specimen, this can lead to improved spatial resolution. Although the present experiments have used 6 MHz beam chopping and $1 \mu\text{m}$ spot size, GHz chopping⁶ could be used with a more finely focused electron beam to obtain $\sim 0.1 \mu\text{m}$ resolution in thin film specimens. Only slight modifications to a commercial scanning electron microscope are needed to chop the electron beam at MHz rates. Secondary electron images can be recorded as well as electron-acoustic images, permitting easy comparisons of microscopic features revealed by the two different techniques. Electron-acoustic images can be viewed at normal SEM scan rates.

Table 1 Contributions of beam spreading d_S and of thermal diffusion d_T to the effective source diameter D^*

	Au	Cu	Al	Si
d_S	0.7	1.4	4.8	5.5
d_T	2.5	2.5	2.1	1.6
D	2.8	3.0	5.3	5.8

* A 30 keV incident electron beam of diameter $d_B = 1 \mu\text{m}$ is chopped at $\omega/2\pi = 6\text{MHz}$ for thick specimens of Au, Cu, Al, and Si. All values are given in micrometres.

Major components of the electron-acoustic microscope are indicated in Fig. 1. Electrostatic deflection plates were located below the second condenser lens of a Stereoscan S4-10 SEM. Sinusoidal and d.c. voltages on the deflection plates were adjusted to produce square-wave beam current. Lenses were adjusted for maximum peak current at the specimen, typically $10 \mu\text{A}$, with a 1.2 mm final aperture and 30 kV accelerating voltage. In most cases a 12 mm diameter, x-cut quartz 6-MHz transducer crystal was bonded with 'Durofix' cement to the bottom surface of a threaded metal plug, as in Fig. 1b, and the specimen to be examined was bonded onto the top of the plug and connected to earth through it. The transducer output, after amplification, rectification and black level suppression, controlled the intensity of the display cathode-ray tube.

Electron-acoustic images obtained with the beam striking either an aluminium plug (Fig. 1b) or the upper electrode of the transducer (Fig. 1c) consisted of irregular arrangements of bright and dark patches of about 0.5 mm size, which moved

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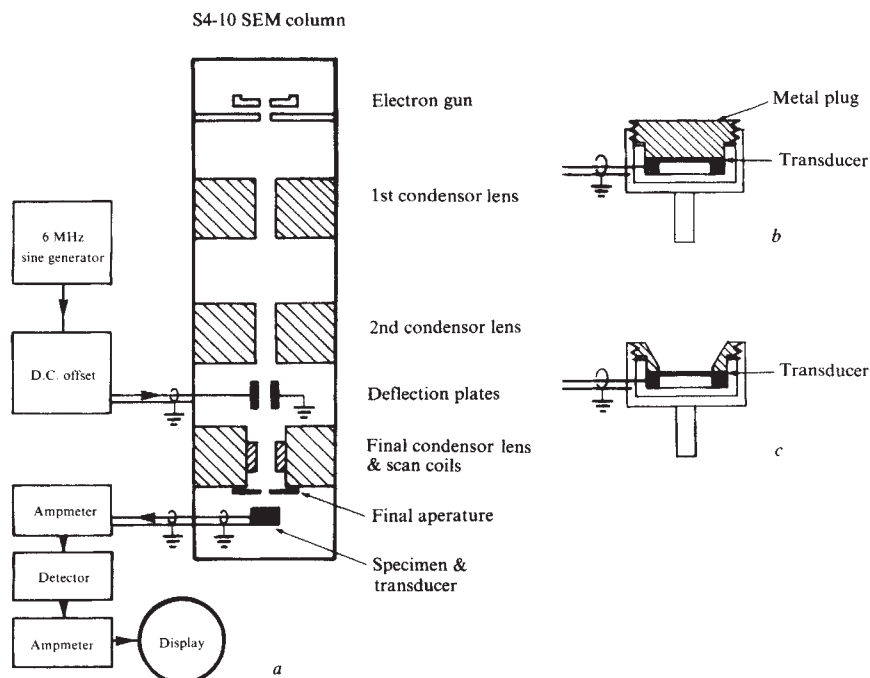


Fig. 1 Major components of the electron-acoustic microscope (a); and cross-section sketches of the transducer assembly, with the crystal bonded to the bottom of a metal plug (b), and without the metal plug (c).

about as the frequency was swept ± 25 kHz. This contrast must have resulted from interferences involving reflected acoustic waves. Because the instantaneous transducer output is proportional to the average normal compression of the crystal, considerable cancellation is expected to occur for either of these arrangements.

Using a plug made from Invar, a low thermal expansion ($\alpha < 2 \times 10^{-6}$) alloy, instead of Al ($\alpha = 23 \times 10^{-6}$), greatly reduced the signal strength. A composite Invar-Al plug was also examined; electron-acoustic and secondary electron micrographs are shown in Fig. 2a. In the electron-acoustic micrograph the Invar is dark and the Al is covered with bright patches. These results are consistent with the contrast between Invar and Al being caused primarily by the larger thermal expansion of Al.

Electron-acoustic and secondary electron micrographs of Fig. 2b show a small area of an integrated circuit chip. In both micrographs the Al conductor lines are bright and the Si substrate, partially covered with silica, is dark. The contrast in parts of the secondary electron image is due to variations of the thickness of the silica, but this contrast is not so visible in regions covered by conductor lines. However, in the electron-acoustic image the brightness of the conductors and of the substrate is reduced over steps in the silica.

The resolution of such electron-acoustic micrographs is essentially the diameter of the region in which elastic waves are

produced by periodic electron beam heating and is determined by (1) the incident beam diameter d_B , (2) the extent of beam spreading in the specimen d_S , and (3) the thermal diffusion length d_T . For the present experiments $d_B \approx 1 \mu\text{m}$, including degradation caused by beam chopping. The penetration and spreading of the electron beam depend on accelerating voltage and on specimen composition and thickness. Values of d_S in Table 1 are estimates for thick specimens calculated with the simplified energy loss expression⁷

$$d_S(\mu\text{m}) = E_0^{1.43}/10\rho \quad (1)$$

where ρ is the density (g cm^{-3}) and E_0 is the incident electron energy (keV). Together, d_B and d_S determine the size of the region of energy disposition. Thermal diffusion further enlarges the source size by about the thermal diffusion length $d_T = (2K/\rho C\omega)^{1/2}$, where K is the thermal conductivity, C is the specific heat, and $\omega/2\pi$ is the frequency⁸. Values of d_T for several materials are given in Table 1, together with resulting estimates of total effective source diameters

$$D = (d_B^2 + d_S^2 + d_T^2)^{1/2} \quad (2)$$

This form of summation is commonly used in electron optics to estimate the effective beam diameter resulting from various lens aberrations (see, for example, equation 11 in ref. 9). For our

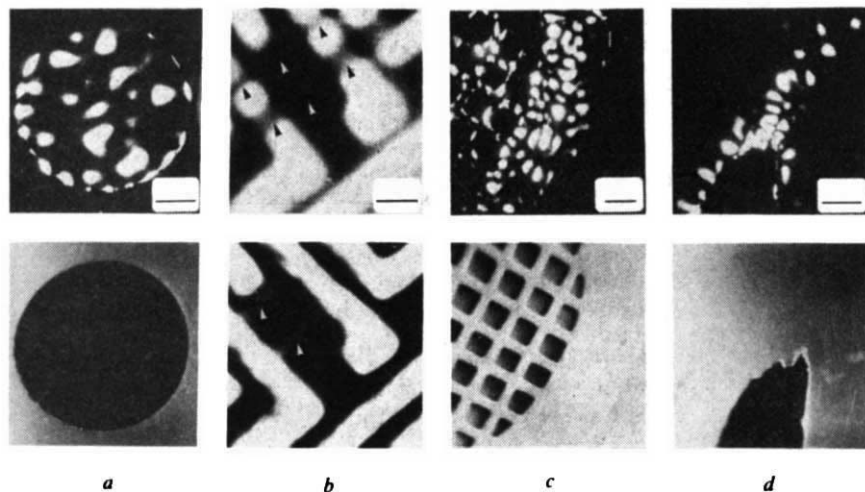


Fig. 2 Electron-acoustic (top) and secondary electron (bottom) micrographs: a, Al rod in Invar plug, scale bar, 0.5 mm; b, integrated circuit chip (arrows indicate steps in silica), scale bar, 10 μm ; c, Cu grid on Cu plug, scale bar, 0.1 mm; d, hole in Al plug, scale bar, 0.5 mm.

experimental conditions, electron-acoustic micrographs are, therefore, expected to have resolutions of 3–6 μm , that is, much smaller than the wavelength $\lambda \approx 1 \text{ mm}$ for longitudinal acoustic waves.

Contrast in electron-acoustic images involves both generation and propagation of ultrasonic waves. For simple cases of elastic wave generation by transient surface heating, White has shown that the stress amplitude for a given absorbed power density is proportional to $\zeta = B\alpha(K\rho C)^{-1/2}$, where B is the bulk modulus⁸. Values of ζ for Invar (<1.0), Al (3.1), and Si (1.1) (in units of $10^7 \text{ dyn s}^{1/2} \text{ cal}^{-1}$) are qualitatively consistent with contrast in electron-acoustic images of Fig. 2a,b.

Although contrast for flat, homogeneous specimens seems to depend mainly on ζ , under some circumstances other factors are also important. Thermoelastic expansions produced by the chopped electron beam are more effective in producing ultrasonic signals when they are well matched to the frequency and spatial characteristics of normal vibrational modes of the material being examined. For example, flexural modes of the 13- μm thick Cu grid in Fig. 2c are more efficiently excited than are vibrations in the copper plug to which the grid is glued. Although acoustic signals passing from the grid through the plug to the transducer are somewhat attenuated in the glue bond, the average signal from the grid is stronger than that from the plug. The bright spots on the electron-acoustic image are the antinodes of the 6-MHz normal mode pattern.

An example of contrast from subsurface features is shown in Fig. 2d. The specimen was a 6-mm high Al plug with a 1.6-mm diameter hole intersecting its upper surface at 6.5°. Only the exit hole is visible in the secondary-electron image. However, in the electron-acoustic image a collection of bright spots of high acoustic signal strength follow the course of the hole until it reaches a depth $z \approx 0.1 \text{ mm}$ below the surface, about 20 times the source depth $D_z = (d_s^2 + d_t^2)^{1/2}$. The magnitude of thermoelastic expansion will not be affected by subsurface features which are well below this source depth. The subsurface hole is seen in the electron-acoustic image mainly through its effect on the propagation and interference of ultrasound. However, sensitivity to subsurface features at depths $z \gg D_z$ is expected to be poor in the present experiments, because the acoustical wavelength λ is much larger than the source diameter D , producing a strongly diverging acoustic wave. Subsurface sensitivity and resolution should be improved by using a much higher frequency so that $\lambda \ll D$.

These results indicate that electron-acoustic microscopy can be used to examine some thermal and elastic properties of solids on a truly microscopic scale. This capability should be particularly useful in studies of defects and inhomogeneities in amorphous solids and in investigations of biological materials. Examination of specimens which are susceptible to electron beam damage may require using lower accelerating voltages and coating specimens with thin, electron opaque films.

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Note added in proof: Examples of lower resolution electron-acoustic images at 100 kHz have recently been reported¹⁰.

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Polymerization of phosphazene crystal by plasma-exposure

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Attempts to polymerize hexachlorocyclotriphosphazene (PNCI_2)₃ by high-energy irradiation methods in the solid state have failed to give a significant amount of the polymer. We report here that (PNCI_2)₃ crystals can be polymerized quite easily when exposed to a low-pressure radio-frequency plasma. The polymerization takes place almost simultaneously with the plasma-exposure. The monomer conversion increases with the plasma power as well as plasma-duration. The product is a white, rubber-like material having a similar appearance to the elastomer obtained by thermal polymerization. The IR spectrum shows the same backbone structure as that obtained by thermal polymerization. It has also been found that post-polymerization takes place at elevated temperatures following a brief exposure (1–5 min) of plasma onto phosphazene crystals.

During the past decade, the chemistry of poly(phosphazene) has undergone significant development, following the demonstration that (PNCI_2)₃ could be polymerized thermally to a soluble high polymer¹ and the chlorine atoms substituted by various organic groups². The presence of phosphorous and nitrogen in the backbone of these polymers causes the wide range of physical and chemical properties and their high temperature resistance³.

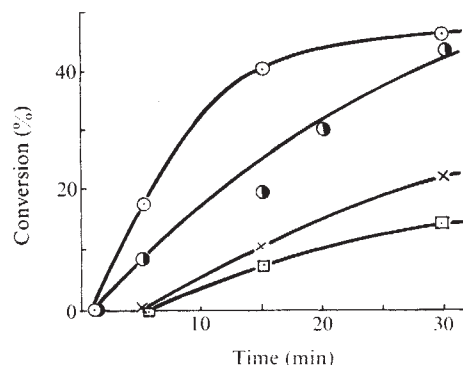


Fig. 1 Monomer conversion versus plasma duration for the polymerization of (PNCI_2)₃ at different power levels. Plasma power: $\circ$, 110W; $\bullet$, 90W; $\times$, 70W; $\square$, 50W. The plasma was sustained at 0.1 torr and room temperature.

(PNCI_2)₃ is usually polymerized by heating the molten monomer *in vacuo* at temperatures of 250–300 °C for 1–2 days (ref. 4). Below these temperatures, monomer conversions of only a few per cent conversion per day are observed. An attempt⁵ to polymerize this cyclic compound in the solid state by γ -radiation has failed to give any significant amount of polymer at dosages, up to 10^6 – 10^8 rad. Irradiation by 4-MeV electrons with a dose of 10^9 rad at –70, 25 and 120 °C *in vacuo* gave only 1–2% of an insoluble product, in spite of the high dose⁶. The only example of solid-state polymerization of (PNCI_2)₃ is that observed using 50 keV X-ray radiation⁷. A maximum of 10% conversion of the monomer to an elastomeric polymer was

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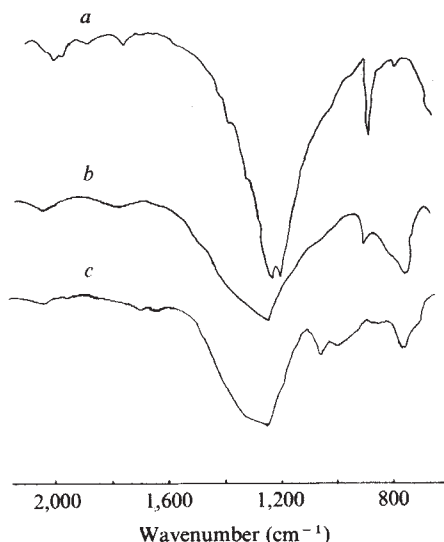


Fig. 2 IR spectra of $(\text{PNCl}_2)_3$ (a) and polymers obtained by thermal polymerization (260 °C, 15 h) (b), and plasma exposed polymerization (110W, 30 min) (c).

observed when the monomer was maintained near the melting point (114 °C) and irradiated for 40 h at a dose rate of 1.2 Mrad h^{-1} .

We have now found that $(\text{PNCl}_2)_3$ crystals can be polymerized quite easily when exposed to the effects of a low-pressure radio-frequency plasma. Plasma exposure was carried out on sublimed $(\text{PNCl}_2)_3$ crystals (Nippon Seika Chemistry Incorporated) placed in a glass ampule which was inserted between a pair of parallel-plate electrodes connected to an RFG-200 radio-frequency generator (Samco International Incorporated), operating at 13.56 MHz. The ampule was evacuated and a room temperature glow discharge was initiated in the phosphazene vapour remaining in the ampule. Following plasma-exposure, the samples were washed thoroughly with *n*-hexane to remove any unpolymerized material.

The polymerization of phosphazene takes place quite rapidly on plasma exposure, and the monomer conversion increases with the plasma power as well as the plasma-duration (Fig. 1). An oily product is formed in the very initial stage of the polymerization. The final product, however, is a white, rubber-

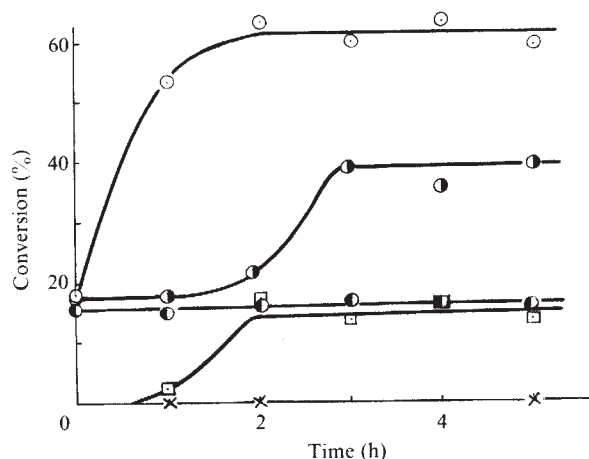


Fig. 3 The effect of time on the postpolymerization of $(\text{PNCl}_2)_3$. Plasma power, 110W; plasma duration and temperature of postpolymerization: $\circ$, 5 min, 220 °C; $\bullet$, 5 min, 180 °C; $\blacksquare$, 1 min, 180 °C; $\times$, no plasma, 220 °C.

like material having a similar appearance to the elastomer obtained by thermal polymerization. Figure 2 shows IR spectra of the phosphazene polymers obtained by plasma exposure and thermal polymerization, and demonstrates that the same backbone structure is obtained by both polymerization methods. Cross-linking might occur through labile chlorine atoms during the plasma exposure, because the observed chlorine content in the polymer decreased slightly, relative to that in the monomer, and a new absorption band attributed to P-O bond appeared near $1,040 \text{ cm}^{-1}$. Postpolymerization has been found to occur at elevated temperatures following a brief exposure of the phosphazene crystals. As shown in Fig. 3, $(\text{PNCl}_2)_3$ exposed to a plasma for 1 or 5 min undergoes postpolymerization up to certain level at 180 or 220 °C, whereas no polymerization takes place in these conditions for $(\text{PNCl}_2)_3$ without plasma exposure.

A mechanism for the polymerization of $(\text{PNCl}_2)_3$ under the effects of a low-pressure plasma has not yet been determined. Thermal polymerization is considered to be a minor component, however, as extensive polymerization takes place when the gas temperature in the plasma was maintained below 160 °C or the phosphazene crystals were kept at temperatures as low as -20 °C.

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Configuration at C-24 in steranes and sterols

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Most marine and terrestrial organisms and aquatic sediments contain sterols with an alkyl group at C-24. Assignment of the configuration at this position has important biosynthetic, taxonomic and geochemical implications¹⁻³; for example, it is required to elucidate biosynthetic pathways responsible for alkylation^{1,2}, and to reveal sources of sterols in species where there is no *de novo* synthesis^{4,5} and in recent sediments^{3,6}. Similarly, routine separation of the C-24 isomers of steranes in sedimentary rocks (formed from sterols present at the time of deposition) should provide information on the nature of the contributing organisms and the isomerization reactions thought to occur in steranes with increasing depth of burial and the associated temperature rise^{7,8}. Using an extension of gas chromatographic (GC) methods applied previously to the separation of stereoisomers of acyclic isoprenoid acids⁹ and alkanes¹⁰, we report here (1) the complete separation of (24*R*)-24-methyl-5 α -cholestane (I) from its C-24 isomer (II in Fig. 1), the partial separation of (24*R*)- and (24*S*)-24-ethyl-5 α -cholestane (III and IV), (2) the application of these separations to a study of the C-24 configuration in the steranes of a sedimentary rock and a petroleum and in those from reduction of the sterols of a marine coccolithophorid, and (3) similar separations of the corresponding 3 β -OH saturated C₂₈ and C₂₉ alcohols as acetates (stanol acetates).

To date, assignment of the C-24 configuration in steroidal compounds has relied on X-ray crystallography^{3,6}, differences in melting points¹¹, optical rotation measurements^{12,13} or chemical shifts in ¹H nuclear magnetic resonance (NMR) spectra¹⁴. These

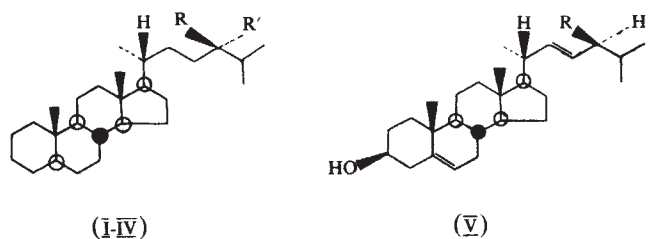


Fig. 1 Structural formulae of C-24 alkylated steranes (I-IV) and major free sterol of *E. huxleyi* (V). (I), R = CH₃; R¹ = H. (II), R = H; R¹ = CH₃. (III), R = C₂H₅; R¹ = H. (IV), R = H; R¹ = C₂H₅.

methods typically require a few milligrammes of the isolated compound and their application to studies of the components of small quantities of complex mixtures is limited.

Chromatographic separation of C-24 isomers of sterols or steranes has proved difficult¹⁵⁻¹⁷. The only report describes partial separation of milligramme quantities of (24*R*)-24-methyl-5 α -cholestane (I) and its 24*S* isomer (II) on alumina¹⁸. In these conditions, previous separation of coeluting steranes is required⁷. Figure 2a shows the partial chromatogram of a 50:50 mixture (quantitated accurately by spiking with 5 α - and 5 β -cholestane) of (24*R*)- and (24*S*)-methyl-5 α -cholestanes ((I) and (II)) obtained with a Carlo Erba Fractovap (series 2151) chromatograph fitted with a 100 m $\times$ 0.24 mm glass column, roughened and deactivated by the barium carbonate technique of Grob *et al.*¹⁹, and coated statically²⁰ with a dichloromethane

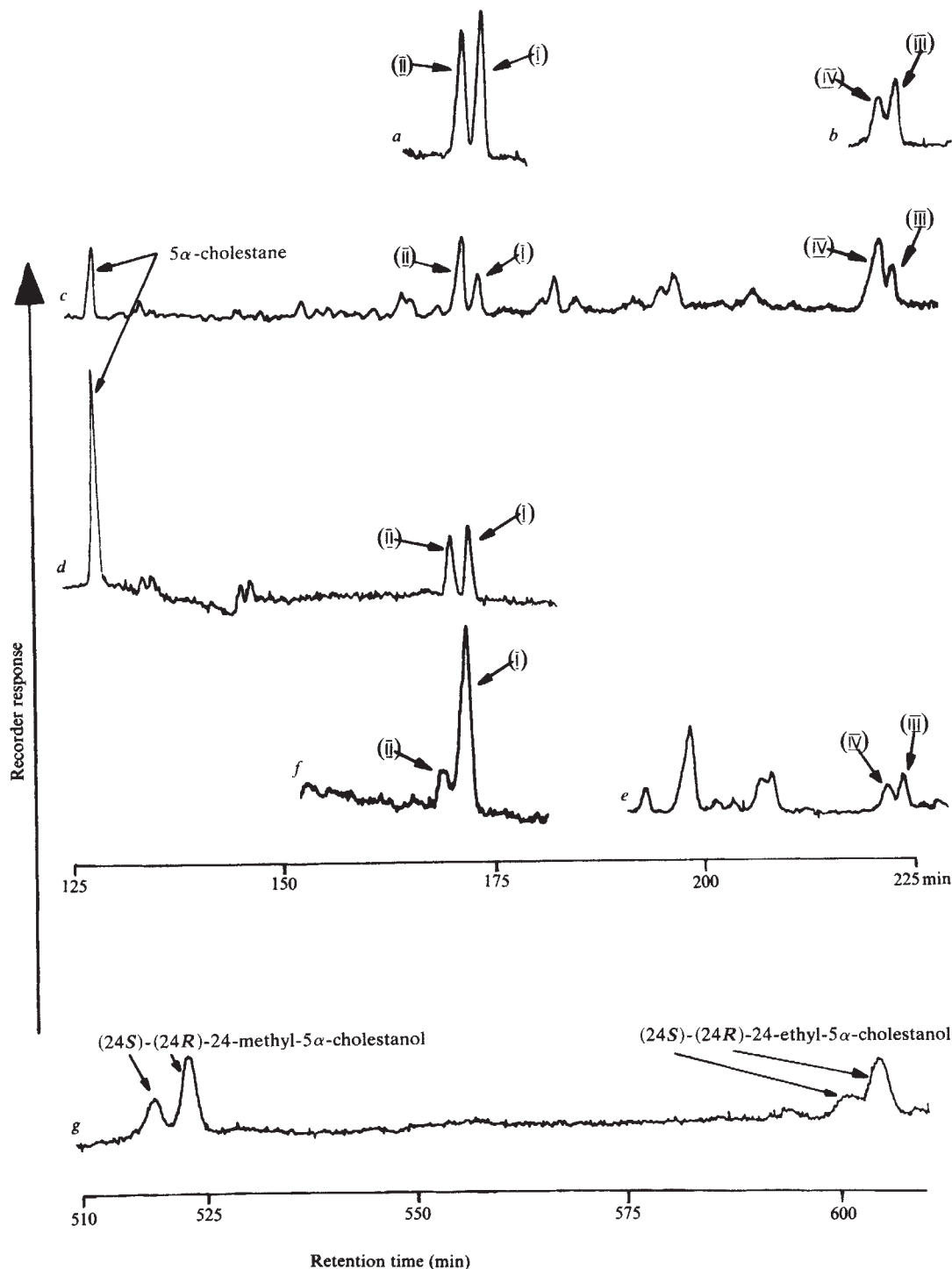


Fig. 2 Partial gas chromatograms of: a, 50:50 mixture of (24*R*)- and (24*S*)-24-methyl-5 α -cholestanes; b, 50:50 mixture of (24*R*)- and (24*S*)-24-ethyl-5 α -cholestanes; c, Green River shale branched and cyclic alkanes; d, thiourea adduct of Rozel Point crude oil branched and cyclic alkanes; e, thiourea non-adduct of Rozel Point crude oil branched and cyclic alkanes; f, steranes from reduction of the free desmethylsterols of *E. huxleyi*; and g, 5 α -stanols (as acetates) from a recent sediment. (For conditions, see text.)

solution of diethylene glycol succinate (DEGS) and polyethylene glycol succinate (PEGS) (75:25; wt:wt) to produce a 0.06 μm film; H_2 was the carrier gas (2.3 kg cm^{-2} back pressure $\sim 7.5 \text{ cm}^3 \text{ min}^{-1}$); the oven was programmed from 40 to 160 $^\circ\text{C}$ at 4 $^\circ\text{C min}^{-1}$ and then maintained at 160 $^\circ\text{C}$. Figure 2b shows partial separation of a 50:50 mixture of (24*R*)- and (24*S*)-24-ethyl-5 α -cholestane ((III) and (IV)) (quantitated as above) in the same conditions. Elution orders were established in both cases by co-injection, with (II) and (IV) respectively, and amounts injected were 5–15 ng of each component. Although both standard mixtures comprise equal amounts of the two isomers, the peak heights are not equal. This is most apparent for the ethylcholestanes (Fig. 2b) where there is a contribution to the second eluting 24*R* isomer peak from the first eluting 24*S* isomer. The areas of the two methylcholestane peaks are equal so the first eluting 24*S* isomer peak is slightly broader (Fig. 2a).

The method has been used to separate the steranes in samples of the Green River shale (Colorado, Eocene) and Rozel Point crude oil (Utah, unknown age), as the C-24 stereochemistry of certain steranes has been assigned previously by $^1\text{H NMR}$ ⁷. Steranes whose nuclear and C-20 stereochemistry differs from that of the presumed precursor sterols have been reported from sedimentary rocks and petroleum^{7,8,17}, but discussion is limited here to those which are isomeric at C-24 and which have the same configuration as the original sterols at all other chiral centres (that is, 5 α , 8 β , 9 α , 14 α , 17 α (H), 20*R*). GC analysis of the branched and cyclic alkane fraction from the shale sample indicated a 75:25 mixture of (24*S*)-24-methyl-5 α -cholestane (II) and the 24*R*-isomer (I), and a 75:25 mixture of the 24*S* C₂₉ sterane (IV) and its C-24 isomer (III; Fig. 2c). These ratios are the same as those obtained independently by $^1\text{H NMR}$ ⁷. The branched and cyclic alkane fraction of the crude oil was too complex for analysis without further fractionation and was subjected to thiourea adduction (twice)²¹. As expected²², the 24-ethyl-5 α -cholestanes were essentially not adducted, whereas 24-methyl-5 α -cholestanes were almost completely adducted. GC analysis of the adduct and non-adduct showed, as determined previously by $^1\text{H NMR}$, the 24-methyl- and 24-ethyl-5 α -cholestanes to be present in 50:50 mixtures of 24*R* and 24*S* epimers (Fig. 2d, e) when compared with the mixtures of the standards. Adduction of the standards revealed no detectable fractionation at C-24.

Thiourea adduction is required for the analysis of the steranes of most shales and oils. The non-adduct is, however, sometimes too complex for direct GC analysis of the C-24 configuration in 24-ethyl-5 α -cholestanes and problems of co-elution with other components are occasionally encountered in evaluation of the 24-methyl-5 α -cholestanes in the adduct. Analysis of the 24-methyl-5 α -cholestanes (in conditions of partial separation) from a suite of Toarcian shales from the Paris Basin shows a preference for the (24*S*) isomer (60:40 in favour of 24*S*) in samples with a mild thermal history, which is lost on increased burial depth⁸. The preservation of a preference for 24*S* components in those shales with a mild thermal history may relate to the sterols of the source organisms. Most algae and fungi seem to produce 24 β (24*S* where the side chain is saturated) sterols, whilst most higher plant sterols are 24 α ^{1,2}. However, there are exceptions and plants can contain both configurations^{23,24}.

The C-24 stereochemistry in sterols is related to alkylation reaction mechanisms^{1,24}. Biosynthetic, taxonomic, and marine studies would clearly benefit from a routine method of separating C-24 isomers and which could be applied to the small quantities of complex mixtures commonly encountered. Hydrogenolysis/hydrogenation of two sterols, (24*S*)-24-methylcholesta-5,22*E*-dien-3 β -ol and (24*R*)-24-ethylcholesta-5,22*E*-dien-3 β -ol, by bubbling H_2 through a suspension of the sample ($\sim 50 \mu\text{g}$) and PtO_2 (1 mg) in glacial acetic acid (5 cm^3) containing perchloric acid (1 drop) produced (after 4 h) yields of $\sim 60\%$ of sterane. The 5 α -stanol acetate ($\sim 20\%$) and the free 5 α -stanol ($\sim 20\%$) were also produced, but no unreacted sterol was recovered. GC analysis of the steranes showed evidence of a small extent of isomerization during the reaction, and a ratio of

4.2:4.5:1 of the two C-24 isomers in favour of the original configuration was observed. This minor extent of isomerization has already been noted by $^1\text{H NMR}$ analysis of the products of hydrogenation of certain sterols²⁵. No isomerization was detected when sterols with no side chain double bond were hydrogenolysed/hydrogenated.

The technique of analysis of the steranes resulting from hydrogenolysis/hydrogenation has been applied to the study of the major free sterol of the microscopic marine coccolithophorid, *Emiliania huxleyi*, family Gephyrocapsaceae²⁶. This ubiquitous marine alga^{27,28} has given rise to extensive calcium carbonate (coccolith) deposits in many deep-sea areas, including the Black Sea²⁹. It is remarkable in that it biosynthesizes a novel carotenoid, 19'-hexanoyloxyfucoxanthin³⁰ and C₃₇–C₃₉ straight chain alkenes and alkenones which are abundant in many sediments^{31,32}. Gas chromatographic/mass spectrometric analysis of the free desmethylsterol fraction as the TMSi ethers, using conditions described previously⁶, showed 24-methylcholesta-5,22*E*-dien-3 β -ol as the major component. The fraction was hydrogenolysed/hydrogenated as above and similar products and yields were obtained. Without purification, the products were analysed by GC on DEGS/PEGS and (24*R*)-24-methylcholesta-5,22*E*-dien-3 β -ol and (24*S*)-24-methylcholesta-5,22*E*-dien-3 β -ol were found to be present in the ratio 4.3:1 (Fig. 2f). The major free sterol of *E. huxleyi* is, therefore, (24*S*)-24-methylcholesta-5,22*E*-dien-3 β -ol (V that is, 24 α).

The latter isomer is the same as that produced by the diatoms, *Phaeodactylum tricornutum*⁵ and *Nitzschia closterium*³³, and it is interesting that the fatty acid composition of *E. huxleyi* is also similar to those of the diatoms³⁴. Phytoflagellate³⁴ *Ochromonas* spp. (Chrysophyceae) produce sterols with the opposite C-24 configuration to that in *E. huxleyi*^{1,11} and such differences may be useful in the classification of many such microscopic algae, which can be difficult²⁶.

Attempts to avoid the reduction step and extend the method to the direct examination of steroidal alcohols have been partly successful. Partial separations of C₂₈ and C₂₉ stanol acetate isomeric pairs were obtained (Fig. 2g) after elution times ~ 9 –10 h (conditions as above, but programming from 40 $^\circ$ to -190°C at 4 $^\circ\text{C min}^{-1}$), but no separation of $\Delta^{5,22}$ sterol acetates isomeric at C-24 has been achieved.

The examples described show clearly the value of the technique in (1) the study of the sterane distributions of sedimentary rocks, and (2) structural studies of sterols in organisms. These preliminary results also suggest that the approach will be useful in assigning the input sources of sterols in sediments. Many of these samples contain complex mixtures with varying degrees and positions of unsaturation, and fractionation by argentation thin layer chromatography³⁵ before reduction should prove useful. Definition of the sources of sedimentary sterols also requires routine determination of the C-24 configuration of sterols biosynthesized and/or incorporated by a variety of individual organisms and the GC approach should allow rapid screening of small quantities of complex mixtures.

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Thermoluminescence dating of volcanic plagioclases

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Thermoluminescence (TL) is a well-established archaeological dating¹ technique but the most common mineral in volcanic rocks, plagioclase feldspar, is affected by 'anomalous fading' which prevents its use for dating lava flows. We describe here how the α contribution to annual dose rate is calculated. Plagioclase ages are compared with those obtained by ¹⁴C, K–Ar and TL dating on quartz. Our samples come from the Chaîne des Puys (Massif Central, France). We conclude that TL is a promising tool for dating continental lava flows in the range 3,000–300,000 yr BP.

The intensity of light emitted during thermal stimulation of a mineral is related to the radiation dose it has accumulated since its last heating (palaeodose). A basic assumption that the electronic trapping states responsible for the TL emission are stable. A routine check, the 'plateau' test, consists of plotting the ratio of the natural TL to the increment of TL induced by a laboratory-added radiation dose (artificial TL, ATL) against temperature; a constant value must be reached above a certain temperature. However, as shown by Wintle², most volcanic plagioclases do not have a plateau in the usual temperature range of study (620–720 K). Thus, the maximum ages obtained by TL are too small by as much as an order of magnitude. This effect was attributed to a spurious loss of electrons from their traps in the 620–720 K temperature region where the mean life of charge carriers should be larger than 10⁵ yr. Bailiff's³ has made promising attempts to avoid anomalous fading using phototransfer.

The study of high temperature TL emission (700–1,000 K) of plagioclases is restricted to the UV spectrum because having to cut off the black-body thermal emission necessitates using a thick glass UV filter. In such conditions, we have already suggested⁴ that glow peaks might be unaffected by anomalous fading.

Figure 1Aa shows the natural TL glow curve of a Labrador aliquot (from Olby flow); Fig. 1Ab is the glow curve obtained when a 16 krad dose is added to another aliquot of the same

weight. The ratio of the natural TL to the increment of TL induced by the added dose is plotted in Fig. 1B. There is no plateau but a tendency for stabilization above 850 K which indicates the onset of a region of different behaviour. As already described for quartz⁵, we try to eliminate the lower temperature TL emission by preheating. Figure 1C shows the plateau test in different conditions of preheating. Figure 1Cc (850 K, for 1 min) is rather flat which indicates that the remaining glow peak is a constant function of temperature.

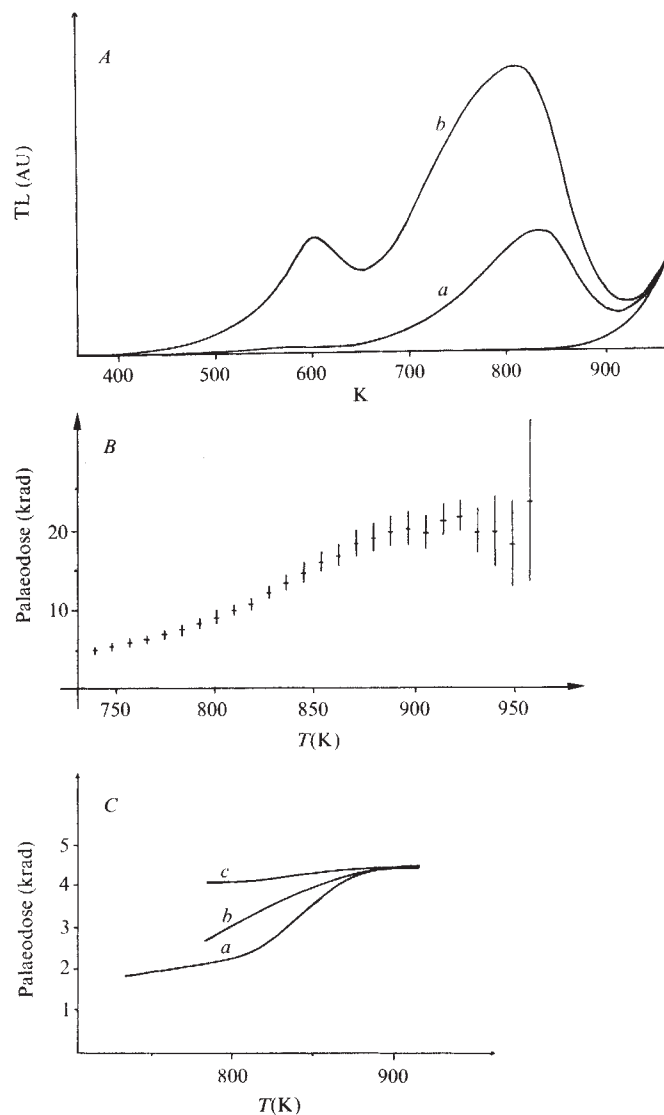


Fig. 1 A, TL glow curves of Olby Labrador: a, TLN; b, TLN + 16 K. B, Plateau test of Olby plagioclase. C, Plateau curves for Saint-Saturnin preheated plagioclase: a, 1 min 735 K; b, 1 min 781 K; c, 1 min 832 K.

Wintle² has pointed out that anomalous fading mainly occurs soon after the sample has received an artificial radiation dose. To check for short-term fading we determined the apparent dose remaining after one year of storage of a lava sample from the Olby flow which had been previously heated to 960 K for 4 h, cooled and γ irradiated at 7.395 ± 187 rad at low dose rate. The apparent measured dose 7.238 ± 293 rad agrees with the deposited dose.

In such conditions there was no evidence of short-term fading; although these two arguments usually allow a mineral to be used for TL dating, they cannot be assumed on a geological time scale. Therefore, we studied lava flows of known age from the Chaîne des Puys (Massif Central, France)⁶.

Table 1 *a*, U, Th and K content of the plagioclase samples; *b*, radiation properties of the plagioclase samples

<i>a</i>	U (p.p.m.)	Th (p.p.m.)	K ₂ O (%)	<i>b</i>	Palaeodose (krad)	Supralinearity (krad)	D_{α} (mrad yr ⁻¹)	D_{β} (mrad yr ⁻¹)	D_{γ} (mrad yr ⁻¹)
St Saturnin	1.70	6.18	1.75	St Saturnin	4.57 ± 0.36	0.60	249.8	161.9	87.1
Laschamp	1.77	9.10	2.14	Laschamp	15.6 ± 1.20	0.40	177.1	193.5	108.2
Puy Laschamp	1.90	6.85	1.78		15.6 ± 1.20	2.30	212.5	165.0	91.9
Olby	2.40	8.83	2.02	Olby	20.20 ± 1.60	0.50	219.4	198.1	114.4
Egaules	1.08	7.20	1.51	Egaules	20.15 ± 1.00	2.70	65.6	136.3	77.9
Montgreleix	1.73	6.28	1.63	Montgreleix	61.50 ± 4.90	9.60	126.8	154.4	85.6
Mazoières	2.00	7.00	1.59	Mazoières	79.50 ± 8.10	Negligible	115.2	153.4	89.8

TL measurements were done on plagioclases ranging from andesine to labrador, carefully extracted from the lava by gentle crushing, and separated by sieving heavy liquid and magnetic techniques. We selected a grain size of 80–125 μm to remove finer microlites and to keep only the microphenocrysts. TL glows were measured with a glass filter (325 nm) which allows a maximum temperature of 1,100 K. The heating rate is generally 5 K s⁻¹ to 10 K s⁻¹, artificial irradiations were carried out with a ¹³⁷Cs γ source (4.02 rad s⁻¹). After preheating, usually at 830 K for 1 min, the plagioclases all presented a single glow peak around 920 K (5 K s⁻¹, heating rate).

The palaeodose is obtained by comparing natural TL with artificial TL, a supralinearity correction is included which results from a non-linear initial increase of the TL signal as a function of the dose (saturation curve). This correction is estimated from the second glow. Annual β and γ dose rates are computed from U–Th and K contents, measured by γ -ray spectrometry with a Ge–Li detector (Table 1*a*), using Bell's data⁷. There is no grain size correction for β radiation from K because the internal K content measured with an EDAX electron-microprobe does not substantially differ from the average K content of the lava. The very small correction, <1% of the total dose, for the β radiation from U and Th families, is neglected. Because of the very high efficiency of α particles in the production of TL in plagioclases (about five times higher than quartz), the α contribution to the annual dose rate is important even when using a grain size of 100 μm . Indeed, the TL induced by α radiation may reach one-third of the total TL as shown in Table 1*b* where the palaeodose, supralinearity correction, and the annual dose rates for α , β and γ radiation are given.

α -dosimetry presents problems mainly because of the short average range of α particles in matter. We must, therefore, know the repartition of the α emitters in the middle where the

plagioclase is naturally irradiated. With a resolution of several microns, comparable with the range of α particles, fission track mapping can represent U distribution in the lava. As seen in Fig. 2 microphenocrysts of plagioclase and olivine near 100 μm have a low U content. Hence the average U or Th concentration of the total lava does not accurately represent the actual concentration seen by the feldspar. As Th and U are strongly linked from a geochemical point of view, we can assume that their distributions are not very different. Thus, a single figure will relate the concentration of U in the plagioclase to the concentration of U or Th of the total rock, for example N_0 , the ratio of the peripheral fission track density to the mean density of tracks in the thin section.

In the lava, a plagioclase grain is submitted to an omnidirectional flux of α particles of various energies. To simulate this flux, we used calibrated laboratory α sources and vibrated the plagioclase grains so that they were irradiated in many positions. For each α particle, omnidirectional experimental α flux was related to the equivalent γ dose which would have produced the same TL; this is termed $\chi(E)$. Up to an energy of 9 MeV, $\chi(E)$ can be fitted by a straight line cutting the χ energy axis at 500 keV. The energy spectrum of α particles from U and Th families, when they are in secular equilibrium (which is checked by our spectrometry measurements) leads to global factors $\hat{\chi}_U$ and $\hat{\chi}_{Th}$ which relate the natural omnidirectional flux to the equivalent γ dose:

$$\hat{\chi}_{U(Th)} = \frac{\int_0^{E_{\max}} \chi(E) \frac{dn}{dE}_{U(Th)} dE}{\int_0^{E_{\max}} \frac{dn}{dE}_{U(Th)} dE}$$

Note that, while assuming $\chi(E)$ to be linear, $\hat{\chi}_U$ is very close to

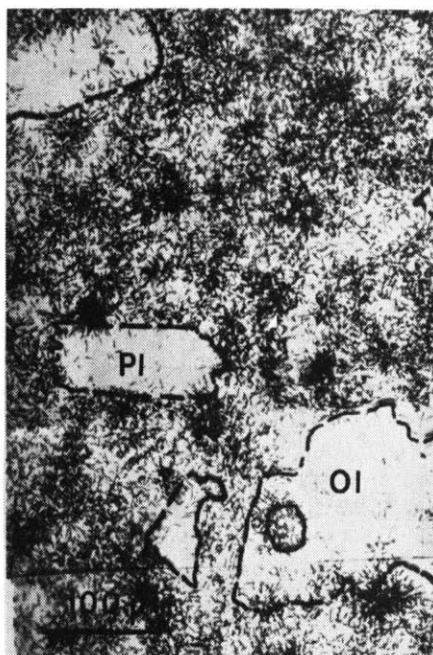


Fig. 2 Photograph of the thin section of St-Saturnin basalt and its fission track mapping. Ol, Olivine; Pl, plagioclase (Labrador).

χ (3.06 MeV) and $\hat{\chi}_{Th}$ to χ (3.40 MeV). Then the contribution to the dose of α particles external to the grain can be written:

$$D = N_0(\hat{\chi}_{Th}\phi_{Th} + \hat{\chi}_U\phi_U)$$

where $\hat{\chi}_U$ and $\hat{\chi}_{Th}$ do not differ much. ϕ_{Th} and ϕ_U are the omnidirectional fluxes, respectively, due to Th and U determined for the total rock. Moreover, we do take into account the U (or Th) generally present inside the grain (<0.2 p.p.m.). The internal contribution to the dose can be written with a similar formula to that of the external α dose but the value of χ is obtained using a grain size of a few micrometres (fine grains technique)¹². In general, the internal α dose rate contribution approximately represents 25% of the α dose (8% of the total annual dose rate).

Table 2 TL dated plagioclase samples compared with conventional techniques

	TL	Conventional	
St Saturnin	8,980 ± 1,150	7,880 ± 350	¹⁴ C*
		8,100 ± 1,100	TL (clay)†
Olby	37,300 ± 3,500	42,000 ± 3,000	TL (Q)‡
		39,000 ± 6,000	U-Th§
Laschamp	31,900 ± 2,850	34,300 ± 3,000	TL (Q)‡
	32,500 ± 3,100		
Egaules	69,580 ± 6,500	75,000 ± 7,500	K-Ar
Montgreleix	163,300 ± 14,500	175,000 ± 13,000	K-Ar
Mazoires	215,800 ± 20,500	242,000 ± 10,000	K-Ar

* Revised from Pelletier *et al.*⁸ using a 5,730 yr half life for ¹⁴C.

† Revised from Huxtable and Aitken⁹.

‡ Revised from Valladas¹⁰ TL dating on quartz, with revised calibration of reference sources.

§ Revised from Condomines¹¹.

|| Revised from Gillot (unpublished work) K-Ar dating on mesostase; Egaules age is an upper limit.

Finally, the estimation of the α dose rate is carried out with an accuracy not much better than 20%.

When crushing the rock the plagioclases are fragmented which leads to an overestimate of χ and an underestimate of the age. For example, if 30% of the 100 μ m plagioclases had an initial size of 200 μ m, the age obtained would be 4% too low¹³. This correction has not been applied to the present data.

Our first results are shown in Table 2, and compared with the ages obtained using more conventional techniques. The agreement of TL dated plagioclases is good and indicates that within our experimental error, $\pm 8\%$, there are no major systematic errors.

We conclude that high-temperature TL emission is not systematically affected by either anomalous fading or by thermal fading and can be used for dating volcanic flows. As saturation doses of plagioclases are around 400 krad, TL allows dating continental volcanics from 3,000 to 300,000 yr BP.

Palaeomagnetism of beach ridges in South Australia and the Milankovitch theory of ice ages

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Although the Pleistocene glaciations continue to attract new explanations¹⁻³, an early explanation in terms of secular variations of the Earth's orbital elements, the Milankovitch theory⁴, has recently gained considerable support^{5,6}. This theory predicts insolation variations with periodicities of ~20,000, 40,000 and 100,000 yr. Its popularity is attributable mostly to deep-sea sediment information on variations of the glaciation-sensitive ¹⁸O/¹⁶O isotopic ratio and the increasingly convincing match of those variations to the Milankovitch climatic curves⁷⁻⁹. Independently, land-based evidence is accumulating in favour of the Milankovitch theory from raised coral terrace¹⁰, loess¹¹, and pollen studies¹². We discuss here evidence for the Milankovitch theory from a South Australian sea-level record.

In the south-east corner of South Australia, a beach ridge sequence extends approximately parallel to the present-day coast line for some 250 km. It is best developed between Robe, on the coast, and Naracoorte, 100 km inland, where eight ridge systems containing 13 well defined parallel beach ridges occur (Fig. 1). The sequence ranges in altitude from near sea level at Robe, to ~100 m at Naracoorte. To the east of Naracoorte the ridges become progressively less well defined but remain recognizable for a further 200 km inland. Offshore from Robe there are at least two more beach ridges representing low sea levels¹³. Detailed drilling of the Robe-Naracoorte sequence¹⁴ has revealed that the ridges are composed of cross-bedded calcarenite, with the lower part of the sequence deposited in beach or near-beach conditions and the upper part in aeolian conditions. In general, the stratigraphy is consistent with a prograding coastal sequence developed on a slowly uplifted region, the beach ridges representing successive high sea-level stands. Sprigg^{13,15,16} has argued that the Robe-Naracoorte sequence and the Milankovitch insolation curves are in good agreement, assuming that the sea-level variations were caused by melting of glaciers during periods of high insolation at critical northern latitudes. Sprigg's comparisons have, however, lacked a time scale based on biostratigraphic or absolute ages. We now date the sequence by polarity reversal, and use this age to test the Milankovitch theory.

Ten of the best preserved ridges were hand sampled between Robe and Naracoorte (Fig. 1). The material was fine- to medium-grained calcarenite composed of a framework of fine abraded biogenic material, quartz, and rare feldspar grains, all coated to varying degrees with iron oxides. Opaque grains, particularly iron sesquioxides, were rare. The cement varied from sparite to microsparite. The sediments ranged from semi-consolidated to well consolidated and their colour varied from yellow to pale reddish brown. Obvious pedogenic modifications, such as colour mottling or pronounced calcretization, were avoided during sampling. Except for the initial work, when a Digico spinner magnetometer was used, all measurements were made with an ScT cryogenic magnetometer. Thermal demagnetization, usually at 50 °C increments, was used to analyse the remanence. Magnetic susceptibilities were determined between heatings to monitor mineralogical changes.

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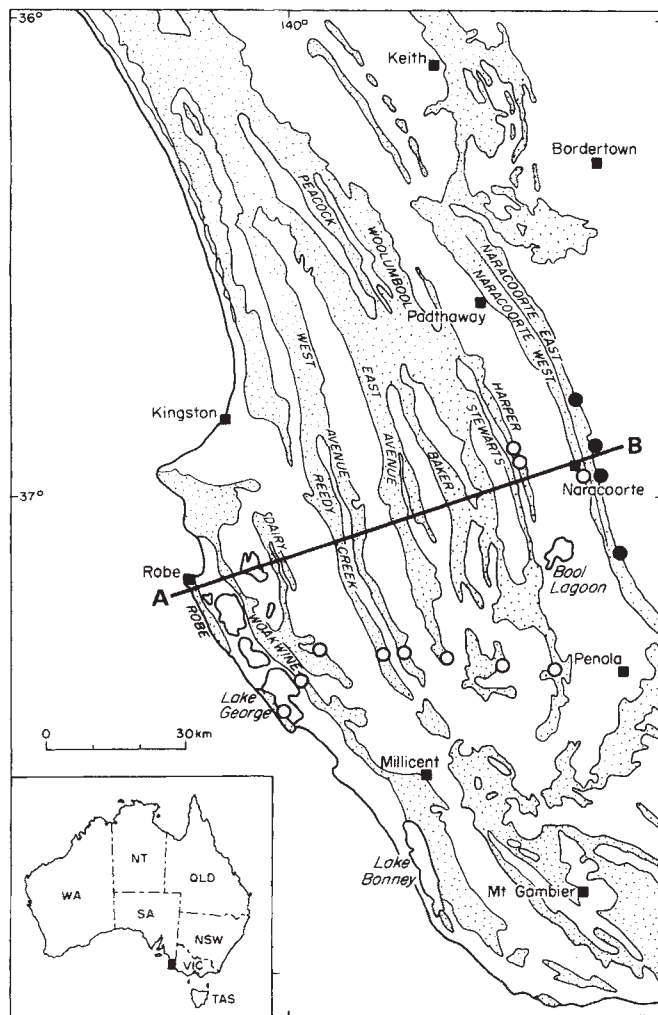


Fig. 1 The beach ridge sequence in southeastern South Australia. Open symbols indicate sampling sites with normal polarity, closed symbols indicate reversed polarity sites.

The NRM intensities ranged from 0.5 to 5 mA m⁻¹. Generally two components of magnetization were observed: a relatively low blocking temperature component which was interpreted as partly a laboratory-storage effect and partly a recent-field effect, and a harder component that exhibited normal polarity for all samples (20) collected between Robe and the West Naracoorte ridge system, and reversed polarity at the East Naracoorte ridge system (Fig. 1). Figure 2 shows thermal demagnetization behaviour of samples collected from ridges adjacent to the polarity reversal. This polarity reversal marks the 720,000 yr Brunhes-Matuyama boundary¹⁷ and provides, for the first time, an age constraint for the ridge sequence. The presence of reversed polarity at the East Naracoorte ridge system was confirmed by sampling at different localities across the ridge and along the ridge axis (nine samples).

The measurements were complicated by an increase in magnetic susceptibility during heatings. This increase was associated with the generation of spurious moments and eventually led to a pronounced magnetic viscosity. In specimens where the onset of the spurious moments was delayed, a third stable magnetization component of very low intensity (0.1–0.2 mA m⁻¹) and high blocking temperature could be isolated. This component showed random directions between different specimens prepared from the same sample.

The most likely origin for the primary remanence is chemical, resulting from processes such as dehydration of iron oxyhydroxides and graining of haematite beyond the critical

superparamagnetic grain volume¹⁸. A detrital origin for the remanence (DRM) is inconsistent with the high-energy beach-aolian depositional environment of the sediments. A post-depositional origin (PDR) seems unlikely because the filling of pore space by the calcite cement would have displaced and misoriented any aligned grains in the matrix. The authigenic haematite occurs as pigment in the cement and as coatings on the surfaces of framework grains, and is related to the processes of reddening of dune sands. The hard component of remanence, characterized by random directions, apparently results from the rare unaligned detrital iron sesquioxide grains in the sediments. While acknowledging that pedogenic processes could overprint and, if sufficiently intense, destroy any Matuyama magnetic epoch remanence, we believe that the intermediate blocking temperature component represents the primary chemical remanence in the samples collected. The absence of obvious pedogenic modifications, such as colour mottling, in the material selected for palaeomagnetic work, suggests that such processes are not significant. The effects of continued graining, post-dating the Brunhes-Matuyama reversal, are more subtle and remain problematic.

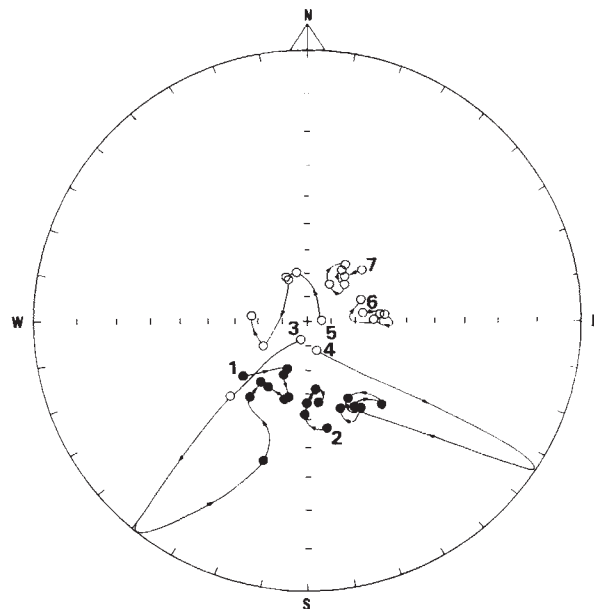


Fig. 2 Remanence directions in beach ridges adjacent to the Brunhes-Matuyama reversal boundary. The diagram shows thermal demagnetization behaviour of seven samples at 100 °C increments commencing from NRM. The arrows indicate directions of increasing heat treatments. Solid symbols correspond to positive inclinations, open symbols to negative inclinations. Samples 1–4 are from two different sites in ridge system 9; samples 5 and 6 are from ridge system 8; and sample 7 is from ridge 7b.

There was probably a time lag between ridge formation and the acquisition of chemical remanence. This time lag would have to be added to the Brunhes-Matuyama reversal date to obtain the true age of deposition. In Victoria, siliceous dunes redden down to 2 m depth within 20,000 yr of deposition. However, more calcareous dunes seem to take significantly longer, possibly of the order of 30,000 yr, for reddening to the same depth (ref. 19 and J. Bowler, personal communication). Bearing in mind that chemical remanence is likely to be acquired before the final stage of reddening has been reached, we may expect the time lag between dune formation and acquisition of chemical remanence to be <30,000 yr. Assuming a remanence acquisition time lag of 30,000 yr, and a depositional time of 30,000 yr for the ridge systems (the average value estimated from the secondary ridge

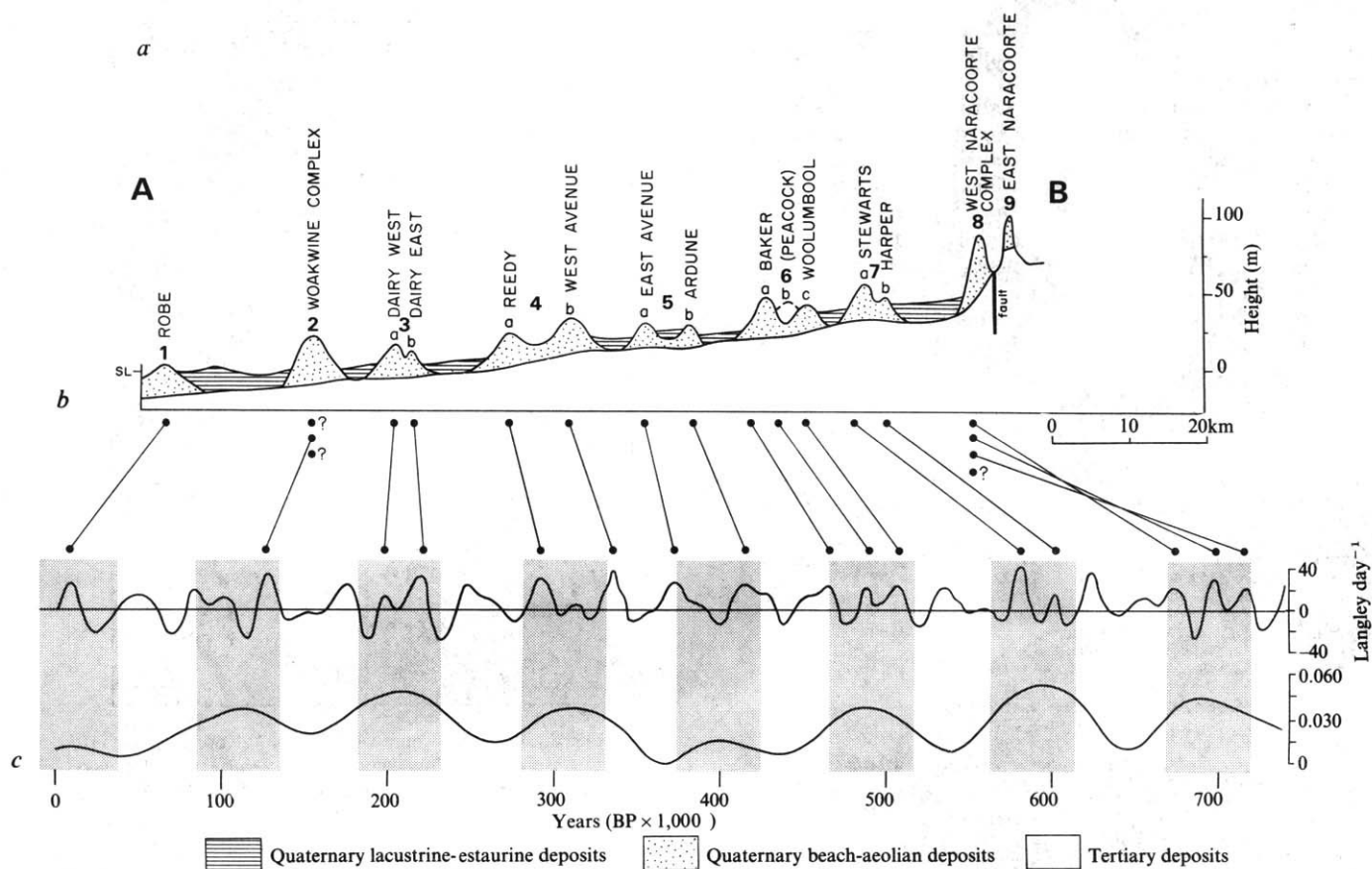


Fig. 3 *a*, Section AB of Fig. 1 through the beach ridges. Ridges 2 and 8 are compound dunes. The Peacock Ridge, which is not intercepted by the line AB, is shown by the broken outline. *b*, Summer insolation in Langley day⁻¹ at latitude 65° N relative to the 1950 AD values (after ref. 20). The stippled intervals indicate interpreted interglacial phases of the (approximately) 100,000-yr climatic cycle. Tentative correlations between ridges and insolation maxima are shown by interconnecting lines. *c*, Variations in orbital eccentricity (after ref. 20).

spacings by linear interpolation between the centre points of the ridge systems which are assumed as a first approximation to be 100,000 yr apart), the mean age of the West Naracoorte ridge system is estimated as 665,000–735,000 yr, depending on the exact location of the Brunhes–Matuyama boundary between those two ridge systems. From the surface expression of the ridges (Fig. 3) and from subsurface drilling information¹⁴, eight high sea-level ridge systems are recognized between Robe and Naracoorte where the sequence seems best differentiated. Assuming that no major ridge systems are telescoped (that the uplift has been continuous, though not necessarily at an absolutely constant rate), the average periodicity of the major ridge systems is obtained as 95,000–105,000 yr (~100,000 yr). A dominant 100,000-yr cyclicity has been found in other climatic records^{7,11,12}. Most of the ridge systems contain more than one dune, suggesting the possibility of shorter-period changes in the sea-level record.

A tentative interpretation of the raised beach ridges in terms of Berger's²⁰ modified Milankovitch insolation curves for latitude 65° N, and the dominant 100,000-yr cyclicity, is shown in Fig. 3. The interpretation is based on the assumptions that the ridges form only during high sea-level stands and that these occur at a particular phase of the 100,000-yr cycle. It also has to satisfy two time constraints: an interglacial (ridge-forming) phase at present, and at 700,000 yr BP (during the deposition of the West Naracoorte complex). The interglacial phases for the sequence are then derived as shown by the stippling in Fig. 3. Note first that the interglacial phases coincide with high-orbital eccentricities. This agrees with the oxygen isotope results⁷, but contradicts the Milankovitch theory that predicts eccentricity dependence in the opposite sense²⁰, and finds only a minor eccentricity contribution to climatic variation. Second, with the

exceptions discussed below there is a one-to-one correlation between the ridges and principal insolation maxima belonging to the 100,000-yr interglacial phases. Third, the subsidiary ridge spacings agree qualitatively with the time intervals between the insolation maxima: the narrow (3 km average) spacings correspond to 20,000 yr intervals and the wide (7 km average) spacings, to 40,000 yr intervals (we assume here, in common with other sea-level studies^{21–23}, a relatively constant rate of tectonic uplift).

Problems with correlation occur in the West Naracoorte and Woakwine complexes, both of which are composed of superimposed dunes^{14,15}. At West Naracoorte where the dunes are stacked against a fault pediment, some doubt exists over the presence of a fourth dune. At Woakwine correlation is possible with only the oldest of the three insolation peaks of the 85,000–135,000-yr interglacial phase: evidence from elsewhere^{21–23} indicates that the high sea-level stands that would correspond to the two youngest peaks of the interglacial phase were well below the first stand. The superposition of Woakwine dunes, indicating repeated transgressions, cannot, therefore, be explained by the Milankovitch theory. Aharon *et al.*²⁴ have recently considered evidence for a non-Milankovitch sea-level high at 120,000 yr that seems to have reached the earliest high mark, and have proposed an Antarctic ice-surge²⁵ as the explanation.

Finally, we note the relevance of the present results to geoidal sea-level changes. The geodetic sea level contains regional irregularities that are subject to change, and any significant geoidal variation would modify the interpretation of sea levels in terms of glacio-eustatic changes²⁶. Although, with the exceptions noted above, the present results are consistent with Milankovitch's predictions of climatic variation and do not require geoidal changes to be invoked, this does not rule out

significant geoidal changes that occur in phase with Milankovitch glacio-eustatic changes, nor major geoidal changes with periods in excess of 700,000 yr.

We conclude that the South Australian ridge sequence contains components related to the Earth's orbital elements. Except for the dominance of the 100,000-yr component and its phase relative to the eccentricity variations, the ridge evidence is consistent with the Milankovitch theory.

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Note added in proof: Recent geochronological results (D. A. Schwebel, personal communication) confirm the interpreted age of the Woakwine complex.

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Quaternary temperatures and precipitation for the north-west coast of North America

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Palynologists utilize present-day pollen rain to interpret the climatic setting of pollen records from Quaternary deposits. Analogues are sought which relate the present with the past. Because climatic conditions at mid-latitudes during the Quaternary were diverse, often ranging from a tundra type at one extreme to a closed forest type at the other, a modern data set should cover the extremes of vegetation and climate expected during this time. For interpreting climatic parameters from Quaternary pollen in land and marine cores, we calculated a pair of regression equations relating modern pollen rain from the Pacific coastal forest and tundra to mean July temperature and mean annual precipitation at a series of sites from the Aleutian Islands to northern California. We describe here how application of these equations to Quaternary pollen profiles from western Washington enabled us to quantify temperature and precipitation over the past ~80,000 yr.

Temperate coniferous forest is the outstanding arboreal vegetation along >4,000 km of the north-west coast of North America between northern California and southwestern Alaska (36-61°N)^{1,2}. In western Washington and southwestern British Columbia its leading constituents, western hemlock, Sitka

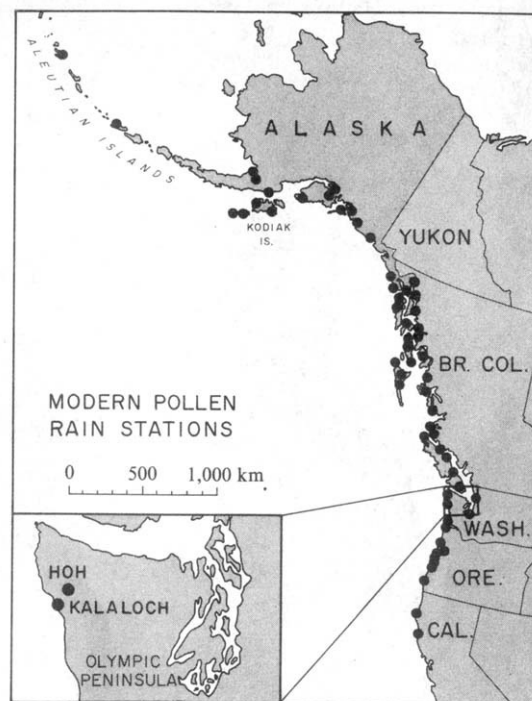


Fig. 1 Distribution of 59 composite sites on the north-west coast of North America showing where modern surface pollen was collected and the location of fossil pollen sites.

spruce, and western red cedar, grow to their greatest proportions. Douglas fir and alder are widely distributed throughout this region chiefly because of disturbance. Southward into California, Douglas fir, pine and redwood rise in proportion in the communities, and a broadleaf sclerophyllous element, consisting notably of oak, tanoak, chinquapin and madrone, enters at the drier inland and southern limits of the forest. Northwestward in Alaska, the hemlock, spruce and cedar become mixed with other species, for example, mountain hemlock, Pacific silver fir and Alaska yellow cedar, which are montane-subalpine to the south. Lowland forest communities increasingly give way to muskeg and, at the same time, lose their complexity, so that on Kodiak Island and on the adjacent Alaska Peninsula, Sitka spruce alone forms the edge of the forest. Beyond this, westward to the Aleutian Islands, the forest is replaced by tundra, at first through an ecotone of shrubby alder and willow.

Optimum conditions of the maritime climate of Washington and British Columbia cause the long growing season and richness of coniferous species that are characteristic of the Pacific coastal forest in this region. Precipitation annually averages near 3,000 mm but decreases to 2,000 mm or less towards the interior; mean July temperature is ~14°C on the coast and ~15°C inland³⁻⁷. Precipitation is heaviest in southeastern Alaska-British Columbia and falls off to less than 1,000 mm in California and southwestern Alaska. July temperatures of 8-10°C in coastal tundra rise by ~6° to ~16°C in California.

Modern pollen rain data are for surface samples from 178 sites at <100 m in elevation located between Attu, furthest west of the Aleutian Islands in Alaska, and the vicinity of San Francisco in California. Data from the sites were consolidated in the form of 59 composite records (Fig. 1). This averaging process ensures that site data represent the broad features of the pollen rain⁸. Surface samples were mostly collected, and analysed by one of us (C.J.H.) to avoid inconsistencies in identification. Thirteen numerically important taxa selected for multivariate statistical analysis from 67 different kinds of pollen identified are pine, spruce, western hemlock, mountain hemlock, fir, Douglas fir, redwood, alder, birch, oak, grass, sedge and composite. Pollen values, percentages of the sum of the 13 taxa at each site, were row normalized and Q-mode, rotated principal

components (factor) analysed⁹. Four resultant pollen factors, which are basically monotypic and account for 93% of the data, are western hemlock, alder, grass and pine. Although pollen rain has many sources of variation^{8,10}, the vegetation of the north-west coast as it varies geographically is the dominant control underlying the distribution of the pollen factors.

A stepwise regression relating the four factors to mean July temperature and annual precipitation yielded a pair of transfer functions (Table 1). Temperature and precipitation data used in calculating the equations were interpolated from 10-yr averages (1968–77) of records from 43 meteorological stations located near sea level between Attu and San Francisco^{3–7}. The western hemlock and pine factors were found to be significant predictors of precipitation, and these along with the grass factor predicted temperature. Alder predicted neither variable at the 5% level of significance. The temperature and precipitation equations, respectively, have standard errors of 13% (1.1 °C) and 19% (620 mm) of the observed ranges. Plotting observed values against predicted values of temperature and precipitation show the residuals to be distributed normally.

The regression equations were applied to fossil pollen percentages (Fig. 1) obtained from a core taken in a bog in the Hoh River valley of western Washington and from a section of biogenic and alluvial sediments exposed in a seaciff some 23 km away near Kalaloch^{11,12}. Pollen derived from the Hoh–Kalaloch sediments provides a continuous data base for the past ~80,000 yr. Chronostratigraphy is based on 14 ¹⁴C dates reaching ~16,000 yr BP in the Hoh core and 14 ¹⁴C dates extending from ~16,000 to >47,000 yr BP in the Kalaloch section.

The resulting curve of mean July temperature for the western Washington coast over the period of record shows a range from ~10 to 15 °C (Fig. 2). The modern mean of 14.5 °C differs from our best estimate of the actual mean by 0.3 °C. The high frequency fluctuations in the Kalaloch values, which partly reflect changes in rates of sedimentation in a fluvio-lacustrine complex, display two relatively warm intervals at ~60,000 and ~30,000 yr BP and the most protracted cold interval during the late Pleistocene. This latter, beginning ~28,000 yr BP and continuing until ~13,000 yr BP, was abruptly terminated by a rapid temperature increase of 3 °C. Amelioration continued until ~8,000 yr BP when temperature reached a maximum for all the time represented by the curve. Subsequent cooling after 8,000 yr BP seems to have been replaced only recently by a warming trend that continues to the present.

Application of the regression equation for mean annual precipitation to the Hoh–Kalaloch pollen data resulted in a curve with a range 1,300–2,400 mm (Fig. 2). The modern annual total is 600 mm less than that measured nearby but is within the error of estimation. Principal features of the curve are the direct relationship shown with temperature during the Pleistocene and the inverse relationship during the Holocene. The pervasive, relatively moist intervals, mostly between 2,000 and 2,400 mm, of the first 50,000 yr of record were replaced ~28,000 yr BP by an extended dry interval. Except for a brief peak in precipitation of 2,200 mm at ~18,000 yr BP, values were on the whole

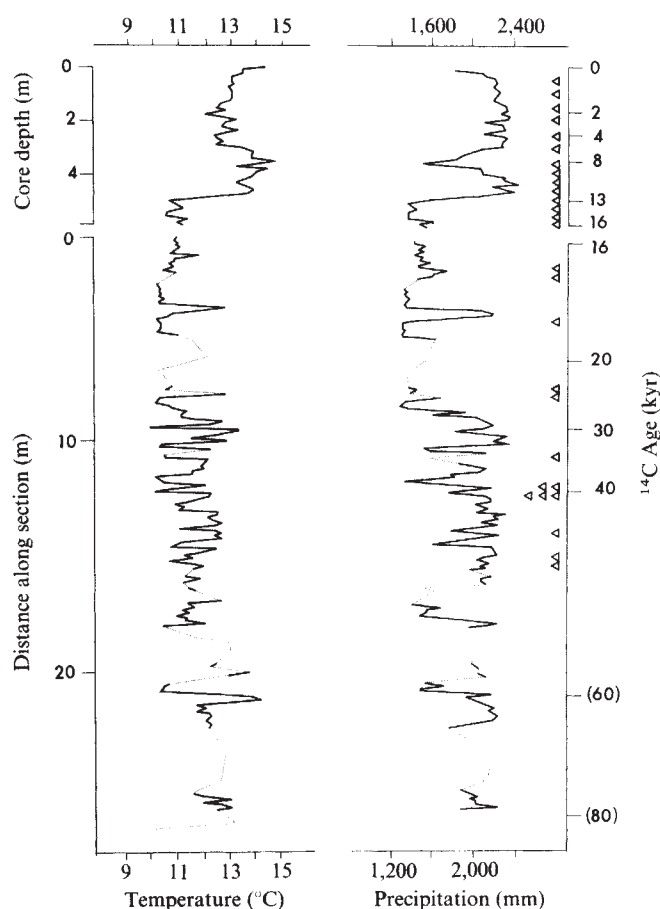


Fig. 2 Mean July temperature and annual precipitation for the past ~80,000 yr represented by the Hoh bog core (upper portion) and Kalaloch seaciff section (lower portion) in western Washington. Arrows indicate ¹⁴C-dated levels; bracketed ages shown are estimates from finite ¹⁴C ages and the rate of sedimentation. Dotted portions of the curves indicate samples with communalities of <0.5 which thus do not correspond strongly to the surface data set.

between ~1,300 and 1,600 mm. At the close of the Pleistocene, precipitation increased synchronously with temperature, reaching a maximum of >2,400 mm. It then decreased, as abruptly as it had increased, to a minimum of ~1,500 mm at ~8,000 yr BP which coincides with the time of the Holocene temperature maximum. Conditions of dryness and warmth ended relatively rapidly, and precipitation since has remained between ~2,200 and 2,300 mm except for a very recent drying trend.

These results extend the application of multivariate statistics to North American Pleistocene pollen records earlier than the late-glacial. Transfer functions applied to pollen data previously yielded temperature estimates for the past 15,000 yr in Minnesota¹³ and the past 5,500 yr in the Northwest Territories of Canada^{14,15}. The curves generated by our data refine trends recorded for the Pacific slope of Washington on semi-quantitative grounds¹⁶ and compare well with records of sea-surface temperature and oxygen isotope stratigraphy of the nearby continental rise^{17,18}. The differences between Holocene interglacial and Pleistocene glacial modes generally agree with models of North Pacific region^{19,20}. The glaciation periods during the Pleistocene were cold and relatively dry with ameliorated moist episodes intervening. During the Holocene, coolness was associated with greater precipitation, and intervals of warmth were drier. This suggests that the prevailing mechanism controlling climate on the north-west coast during the present interglaciation contrasts with the mechanism in effect during glaciation.

Table 1 Transfer function equations for the north-west coast of North America

Variable (factors)	Regression coefficient
Equation NWNA-1 (July temperature)	
X ₄ grass	-5.00737
X ₁ western hemlock	-3.40429
X ₂ pine × pine	-7.62694
X ₃ pine	-6.34598
Intercept	15.02636
Equation NWNA-1 (Annual precipitation)	
X ₁ western hemlock	1,216.99829
X ₃ pine	-735.70166
Intercept	1,152.01180

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Goat Paddock cryptoexplosion crater, Western Australia

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Goat Paddock, a crater slightly over 5 km in diameter (18°20' S, 126°40' E), lies at the north edge of the King Leopold Range/Mueller Range junction in the Kimberley district, Western Australia (Fig. 1). It was noted as a geological anomaly in 1964 during regional mapping by the Bureau of Mineral Resources, Geology and Geophysics and the Geological Survey of Western Australia. The possibility of its being a meteorite impact crater has been discussed¹, although this suggestion was subsequently ignored². Two holes were drilled by a mining corporation in 1972 to test whether kimberlite underlay the structure. Here we report the findings of five days of reconnaissance in August 1979 which established that Goat Paddock is a cryptoexplosion crater containing shocked rocks and an unusually well exposed set of structural features.

Goat Paddock is a circular plain bounded for most of its circumference by steep cliffs 100–150 m high (Fig. 2). To the north, the rim has been eroded, opening a broad connection with the valley of the O'Donnell River. To the south and east, the ground is higher and more rugged, so that a raised rim is not evident. The circularity of the rim is reduced by erosion and by the presence of remnants of crater fill within the wall. Its topography is clearly unusual but not immediately suggestive of a crater.

The two drill holes, one at the centre and one about 1,250 m north-east of the centre, penetrated 211 and 212 m of lacustrine sediments before entering brecciated sandstone. The nearly equal depths suggest the absence of a central peak, which might have been expected as the critical diameter for the transition from simple to complex terrestrial cryptoexplosion craters is 2–3 km in sedimentary strata and about 4 km in crystalline rocks³. The fine-grained nature of the mudstone at the base of the crater fill, immediately above untransported breccia in both drill holes does, however, suggest that there may be a moat in the outer crater floor that acted as a trap for coarse debris. The small depth/diameter ratio is closer to those of larger, complex craters than to those of smaller, simple craters³.

The canyons eroded into and through the walls of Goat Paddock offer some of the best exposures of the upper rim wall and rim flank of any cryptoexplosion crater in the World (Fig. 2). Nearly everywhere in the walls, bedrock has been upturned from its regional dip of 15° NNW to dip away from the crater. Dips reach the vertical near the crater lip and are overturned to the horizontal or inverted outward dips on the rim flank. In some sections, the rim flank appears to be composed of moderately dipping and presumably overturned imbricate plates a hundred metres or so across which are separated by screens of breccia. Intensely disturbed rock reaches as far as 800 m from the present rim crest. At one locality, a fault surface with a dip of 75° towards the crater and with striations pitching 75° separates undisturbed sandstone from overturned sandstone (Fig. 2b). The sense of motion on the fault was not determined; it may be either a thrust fault or a normal fault produced by slumping of the rim into the crater. On the south rim, no overturned flap is

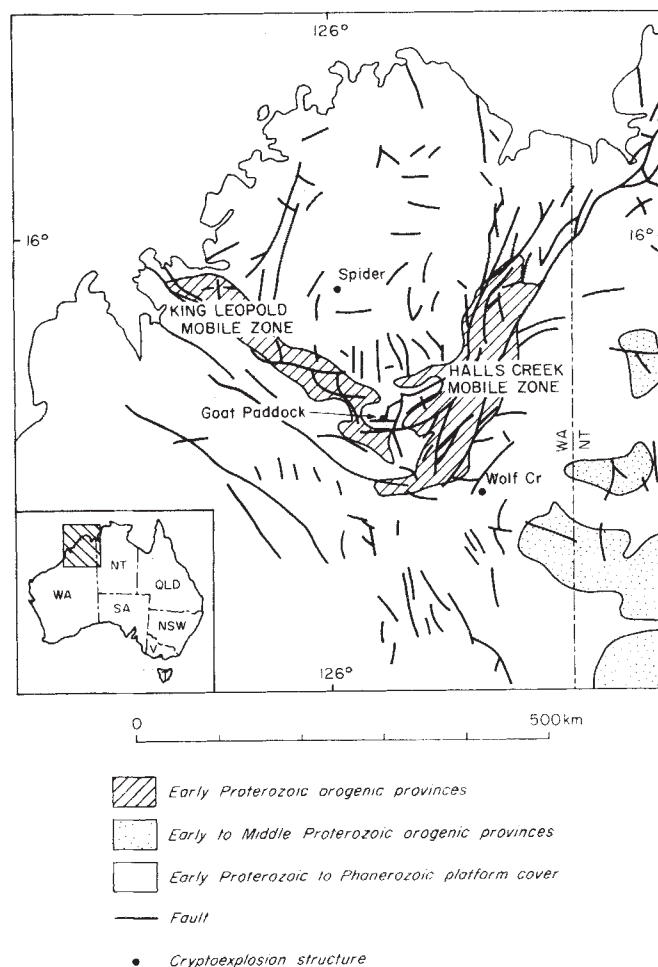


Fig. 1 Locality map of a portion of northwestern Australia showing main tectonic features and cryptoexplosion structures.

apparent, but the autochthonous rock is deformed into at least one open anticline (Fig. 2c).

The disturbed beds show a high degree of fracturing everywhere and grade into breccia inside and particularly outside the rim crest. At many breccia occurrences, attitudes of the larger clasts are close to those of nearby coherent bedrock, and the lithologies are the same, showing that little mixing or long-distance transport has taken place. On the south rim, a series of ridges ~20 m high have fractured bedrock at the base and breccia at the top. No fallout (air-sorted debris) was found on the rim, and we are uncertain whether the breccia at high levels on the rim includes throwout (debris ejected ballistically).

The true crater wall (at the edge of the upturned beds) has a slope of 35°, locally reaching 50° or more. A breccia of unoriented clasts, tentatively interpreted as autochthonous breccia is usually immediately adjacent. This generally grades over a short distance into breccia having a roughly oriented texture, which is apparently talus. Above this breccia is a well-cemented conglomerate with rounded clasts and distinct bedding. The transition is locally gradational, but more commonly conglomerate dipping 20° discordantly overlies talus dipping 35°. The conglomerate, in turn, grades into grit, interbedded grit and siltstone, and finally the mudstone of the crater floor.

Conglomerate (and breccia) occurs not only within the crater bowl but also in depressions outside the crater rim. Some of these depressions appear to be crescentic troughs concentric with the rim. In one section the entire series of overturned plates seems to be displaced outwards to produce a double rim crest, with the gap between the outer edge of upturned bedrock and the inner edge of the displaced plates filled by breccia and conglomerate (Fig. 2b). Other conglomerate bodies appear to be approximately radial rather than concentric, and some of the gorges seem to be exhumed from conglomerate rather than freshly eroded through bedrock. Radial conglomerate bodies may occupy zones opened during the cratering process or they may simply fill pre-cratering valleys. The local topography at the time of the cratering event was probably no less rugged than the present topography and should have affected the details of the crater form.

The level of fill within the crater is close to that of a break in slope of the original crater wall, which may be a junction of the steep wall with the gently sloping outer crater floor or with a bench between the steep wall and floor. Thus, although around most of the circumference early crater fill laps onto the wall or is concealed by fans formed during the present erosion cycle, at several points overturned bedrock crops out as far as a 350 m from the base of the cliff. A small patch of melt breccia was found in one creek between the base of the wall and the innermost bedrock outcrop (Fig. 2c). The substrate is not exposed, but apparently slopes gently, as the larger melted clasts are approximately horizontal.

The central drill hole penetrated 19 m and the northeastern hole 5 m below the crater floor. Core recovery was poor, probably because of abundant incoherent rock floor, but recovered core shows sandstone with well-developed shatter cones or with a rhombic shatter fracturing. A few fragments with poorly developed shatter cones were also found in the breccia at the crater wall. Shock features in quartz (cleavage, planar features, basal deformation lamellae, plastic deformation, reduced birefringence) are abundant in clasts from breccia outcrops. These features culminate in the melt breccia which contains, besides clasts of various lower degrees of shock, twisted sheets of vesicular silica glass, now largely recrystallized to fine-grained quartz. To date, coesite or stishovite have not been found.

More than 20 pollen or spore species were identified in a sample of sediment from a depth of 139 m in one of the drill holes. The assemblage is dominated by proteaceous-like grains. Despite differences, there are sufficient species in common to indicate a correlation with the *Malvacipollis diversus* zone of Stover and Partridge⁴ in southeastern Australia and therefore a probable early Eocene age. This is supported by a very low frequency of *Nothofagidites* spp. and the association of species

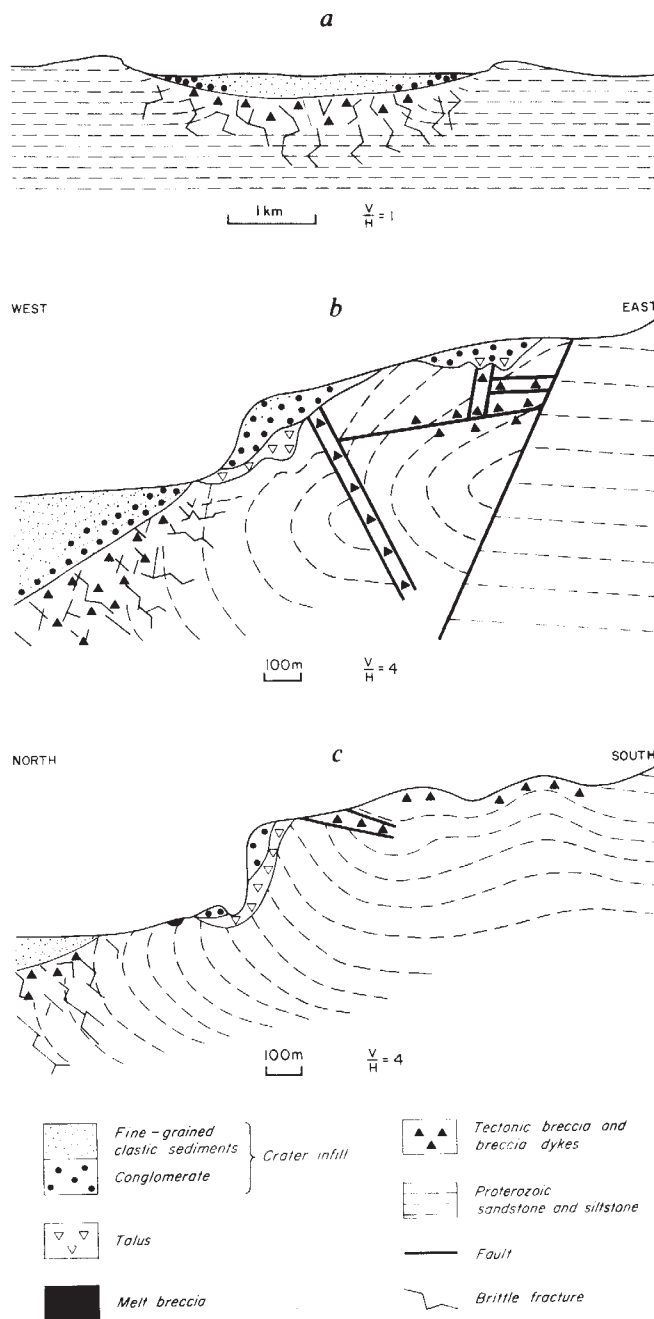


Fig. 2 Cross-sections of Goat Paddock Crater. *a*, Generalized structure; *b*, section along gorge eroded through east wall; *c*, section through south wall. Sections are based on photographs and sketches, and are hypothetical below the level of the crater floor.

such as *Latrobosporites amplius* (Stanley), *Proteacidites leightonii* Stover, *P. confragosus* Harris, and *Triporopollenites ambiguus* Stover.

The orientation of remanent magnetism in the melt breccia is normal and not significantly different from that of the present local field, but samples have not been magnetically cleaned. An Eocene orientation should be recognizable but could have been lost during weathering.

Goat Paddock is clearly a cryptoexplosion crater, of a type generally attributed to meteorite impact. Nevertheless, in the absence of meteoritic material, hypotheses of endogenous origin are defensible. The 875 m diameter Wolf Creek Crater^{5,6}, which is only 160 km to the south-east, is associated with oxidized meteoritic iron, although the association could be coincidental⁷. Wolf Creek seems to be less eroded than Goat Paddock, so an age as old as Eocene and a paired relationship seems unlikely

although not impossible. About the same distance north-west of Goat Paddock is another cryptoexplosion structure, known as 'The Spider' (Fig. 1), which we have also briefly examined. Disturbed and shatter-coned strata occupy an area about 5 km in diameter. The structure resembles that of central uplifts of other cryptoexplosion structures and is apparently exposed at a level considerably below an original crater floor. The depth of erosion indicates an age greater than that of Goat Paddock. The Spider and probably Wolf Creek are in tectonically stable blocks, but Goat Paddock lies near the intersection of the King Leopold and Halls Creek Mobile Zone (Fig. 1), which have been intermittently active through geological time. Adjacent to the King Leopold Mobile Zone is a major province of salic alkaline igneous intrusion^{8,9} dated as early Miocene¹⁰; this province, and an area adjacent to the Halls Creek Mobile Zone, have also recently been identified as areas of major kimberlite intrusion of unknown age.

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Did Iapetus start to open during the Cambrian?

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The geological evolution of the British Isles during the Lower Palaeozoic was dominated by the presence of the Iapetus Ocean. Although the timing of the major events in its later history are reasonably well known, its pre-Ordovician evolution is far less clear because of the absence of rocks of that age in the area of the Iapetus suture. To deduce the early history of the Ocean, circumstantial evidence from rocks originally deposited on the adjacent continental crust is needed. Petrographical evidence, from such rocks in the Dalradian, is discussed here and implies that Iapetus had still not opened by ~670 Myr ago. A dramatic increase in tectonic instability after this date, culminating in the extrusion of the Lower Cambrian Tayvallich Volcanics, is considered to date the continental rupture that led to the formation of Iapetus. This view is in contrast to the assumption that continental break-up must have preceded the earliest Dalradian sedimentation¹ and that the red-bed fluvio-lacustrine sediments of the Torridon Group, dated at ~810 Myr, could be a consequence of this initial rifting².

The Dalradian Supergroup contains a virtually continuous record of largely marine deposition from the late Precambrian (sedimentation starting at least 700 Myr and possibly as long as 800 Myr ago) until the Cambrian or possibly Lower Ordovician. Within this sequence are several psammite formations that are largely composed of mineralogically mature quartzofeldspathic detritus that tells one little about its source area. However,

within the middle Dalradian (Argyll Group) Jura Quartzite are occasional rounded pebbles of what was, before Caledonian metamorphism, ferruginous chert or jasper³. These show a range of textures typical of the oolitic and banded facies of the Proterozoic cherty iron-formations found in early Proterozoic shelf sequences. Furthermore, they are very similar to pebbles described from the Applecross Formation of the Torridon Group⁴ which is known from palaeocurrent and radiometric evidence to have been derived from an area to the north-west of the present Scottish mainland⁵. The mineralogical maturity and absence of all but these very stable clasts, probably recycled from older conglomerates, suggests that the Jura Quartzite source area consisted largely of Proterozoic sedimentary rocks, with only minor first cycle input from the Archean basement. The Scottish 'Torridonian' together with the Eriksfjord Formation⁶ and the Ketilidian sediments⁷ of South Greenland may just be remnants of what was, in late Proterozoic times, a more extensive sedimentary cover over the now exposed Archean basement.

The similarity between the Dalradian and some of the Torridon Group pebbles, together with evidence of a clastic input from a northwesterly direction into some Argyll Group turbidite basins⁸, suggests a northwesterly, largely sedimentary source for Argyll Group psammities. This source must have lain in the area of the present Scotland–Greenland continental margins although it may have stretched even further to the north-west (Fig. 1a).

Most lower Dalradian Appin and Grampian Group coarse-grained sediments are also composed of mineralogically mature quartzofeldspathic detritus without distinctive clasts⁹ that could similarly have been derived from a largely sedimentary source area lying to the north-west throughout the late Proterozoic. The position of the Moine metasediments in this palaeogeographical picture is, however, as unclear as the status of the Moine itself. The ~750 Myr ages from Moine pegmatites have been interpreted as either the date of a 'Moravian' orogenic event¹⁰ or Grenvillian ages partially reset by Caledonian metamorphism¹¹. The lack of petrographical evidence for a nearby orogenic belt in the Appin and Grampian Groups, whose deposition probably spans the period of the Moravian event, is more consistent with the latter hypothesis. However, if the timing of the Moravian event is confirmed, its significant tectonometamorphic effects may have only been felt at depth with its palaeogeographical impact being restricted to mild uplift and erosion of the low grade top of its metasedimentary pile.

The hypothesis of a northwesterly, largely sedimentary, source could well apply to all the Dalradian Grampian, Appin and Argyll Group coarse-grained sediments except for one, the Port Askaig Tillite. This lies at the base of the Argyll Group stratigraphically below the Jura Quartzite and is correlated with the Varangian or Eocambrian tillite found throughout the Caledonian belt and dated in Finmark¹² at ~670 Myr. It contains a suite of granite clasts throughout its outcrop from Banff to Connemara, quite unlike anything found in the other psammities, and must have been derived from a largely igneous and metamorphic source^{13,14}. If the area to the north-west was blanketed by Proterozoic sediments at this time, a southeasterly source is required for the Tillite as previously suggested by Howarth¹⁴ and Spencer¹⁵. This is consistent with the direction of ice-pushed folds from the Tillite in Argyll¹³, Donegal¹⁴ and elsewhere in the Caledonian belt¹⁵. The Midland Valley land-mass, which may have acted as a southeasterly source for the Southern Highlands Group¹⁶ during the Cambrian (Fig. 1c) and was probably of the right composition to supply granite and quartzite clasts, would not have been large enough to nourish the extensive and persistent glaciers of Port Askaig Tillite times. Rather, the glacier source most probably lay in a more extensive area further to the south-east which, on petrographical grounds, could have been in northern continental Europe¹⁵ (Fig. 1b). It is, therefore, most unlikely that there could have been an ocean basin between this presumed source and the present Dalradian outcrop at this time.

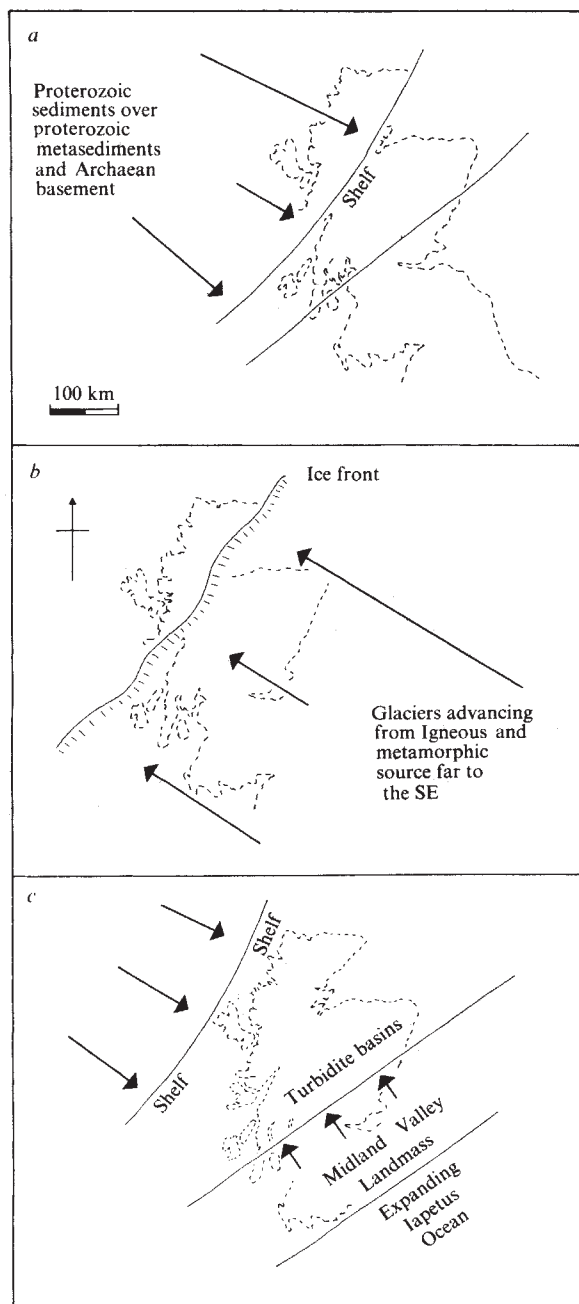


Fig. 1 Location of source areas during Dalradian times, arrows show suggested directions of main sediment influx into the Dalradian depositional area for: *a*, most of Grampian Appin and Argyll Group times; *b*, Port Askaig Tillite times; and *c*, Southern Highland Group times. Position of palaeogeographical elements drawn very schematically with respect to present rock outcrops and without any correction for tectonic shortening. Ice front drawn for one instant in time, the ice limit may have lain far to the north-west.

If Iapetus had not opened by Port Askaig Tillite times, when did it open? Before the deposition of the Tillite, there is little evidence for tectonic instability. Broad, differential subsidence can account for thickness and regional facies variations. However, after the Tillite, major syndepositional faults exerted an increasing control over sedimentation. Rapid subsidence along these faults produced turbidite basins with steep, unstable slopes as exemplified by the Scarba Conglomerate/Easdale Slates⁸, Crinan Grits (A. K. Barraclough, personal communication) and Tayvallich Limestone¹⁷. Finally, eruption of the tholeiitic Tayvallich lavas and intrusion of sills¹⁸ marked the peak of tectonic instability and crustal tension. The rapid basin subsidence is considered to be a precursor of rifting due to

regional crustal tension with the lavas signifying the final break. An alternative hypothesis is that the tectonism and volcanism relate to back-arc spreading following the initiation of subduction. Both are considered feasible by Graham and Bradbury¹⁹ on petrochemical grounds, but the latter is rejected here because of the petrographical evidence discussed above.

The precise dating of the suggested rifting is not possible because of the minimal fossil and radiometric control on Dalradian events. However, the base of the Cambrian probably lies not far below the Tayvallich Volcanics²⁰ and the Argyll Group turbidite basins range from roughly the middle of the Vendian or Eocambrian, say 640 Myr ago, onwards. A period of crustal tension leading to increasing tectonic instability after 640 Myr, culminating in volcanism and final continental break-up around 600 Myr, is therefore indicated. This is in close agreement with an end Proterozoic/Lower Cambrian age for rifting in Newfoundland, suggested originally by Bird and Dewey²¹, on the basis of the stratigraphic relationships of a suite of lavas correlated with the subsequently dated ~605 Myr old Long Range Dykes²². This leaves only ~90 Myr for ocean expansion, about the same as the time available for closure, if subduction started in the Lower Ordovician as suggested by evidence from the Ballantrae ophiolite²³ and final (probably diachronous²⁴) closure took place during the late Silurian to early Devonian²⁵. At, say, a total spreading rate of 4 cm yr⁻¹ this would still produce an ocean 3,600 km across, as wide as is required by available faunal²⁶ and palaeomagnetic²⁷ evidence from the Ordovician. However, the existence of well defined trilobite provinces in Britain does require the existence of an effective barrier by the late Lower Cambrian²⁸, possibly only 20 Myr after the suggested rifting. A faster spreading rate may, therefore, be indicated.

The hypothesis of a late opening for Iapetus has, if correct, several implications. (1) The existence and location of the area of Dalradian sedimentation were unrelated, at least in its early stages, to Iapetus. The Dalradian developed in an ensialic crustal environment¹⁶. (2) Continental rifting subsequently took place to the south-east of the Dalradian basin in an area of more brittle crust. There is, therefore, no stratigraphic mirror image of the Dalradian to the south-east of Iapetus, although because of the possible oblique opening and closing of the Ocean, this cannot be demonstrated until the relative positions of northern and southern Britain during the late Proterozoic are known. This uncertainty also makes it difficult to relate the evidence for late Proterozoic subduction in Anglesey, which may be more closely related to the evolution of a southerly Cadomian Ocean²⁹, to the Dalradian picture. (3) The direct stratigraphic evidence for the timing of rifting, such as plateau lavas, fluvial facies and salt, is not visible in the British part of the Caledonian belt, although it may be buried beneath younger rocks near the suture or have been thrust under the Dalradian during its collision with the Midland Valley microcontinent during the Grampian orogeny³⁰. The presently exposed Dalradian rocks were deposited at least 100 km (possibly several hundred kilometres after allowance for tectonic shortening) away from the rifted continental edge, in a pre-existing marine basin well away from the more obvious effects of continental rupture.

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Equilibrated Nd–unequilibrated Sr isotopes in mantle xenoliths

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Isotopic analyses of mantle xenoliths transported to the surface by alkali basalt eruptions complement data obtained from studying basic magmas. These xenoliths also enable mantle characteristics to be examined before they have been affected by the fractionation processes which occur during magma genesis. The data on ultramafic nodules demonstrate Sr isotopic disequilibrium between mineral phases within a single xenolith^{1–5}. This suggests that isotopic disequilibrium on a grain-size scale may be important in determining the isotopic composition of melts derived from the mantle. However, recent work on carefully cleaned mineral separates from alpine peridotites⁶ and ultramafic nodules² has shown that, in many cases, the highly radiogenic ⁸⁷Sr/⁸⁶Sr found in minerals with low Sr concentrations (olivine, orthopyroxene) can be considerably reduced by leaching the sample in dilute acids before analysis. To investigate the nature of isotopic disequilibria between minerals of lherzolitic nodules, we report here a combined study of the Nd and Sr isotopic composition of two spinel lherzolites from New Mexico and Arizona. These samples were selected from a large number of ultramafic xenoliths for which major and trace element compositions had been determined⁷. The two nodules measured represent two end members of a distinct group of nodules which have major element compositions that would make them suitable parent materials for basaltic melts. These two nodules show no obvious signs of metasomatic alteration or re-equilibration under more than one pressure and temperature regime. Interstitial glass does not occur in either nodule investigated. Hence, the Nd and Sr isotopic composition of these nodules and constituent minerals were expected to provide important constraints on the nature of basalt source regions in the mantle in general, and, in particular, that underlying the American South-West.

Sample KH-1 is a spinel lherzolite from Kilbourne Hole, New Mexico, a recent maar-type volcano in the Rio Grande Rift. Major and trace element compositions of KH-1 were reported by Jagoutz *et al.*⁷. This sample is characterized by considerable depletion of the light rare earth elements (LREE), but still has relatively high Ca and Al abundances (2.45 and 1.89 wt % respectively). KH-1 is coarse granular in texture and, based on petrographical and chemical evidence, was defined as 'primitive'. From sample KH-1, carefully separated and cleaned

clinopyroxene (cpx) and orthopyroxene (opx) separates, as well as a leached whole rock (WR) aliquot and its surface leachant, were measured.

Sample SC-1 is from the recent, xenolith-laden, alkalic magmas of San Carlos, Arizona. As reported⁷, SC-1 has an almost flat relative REE pattern with many other refractory elements also in nearly unfractionated 'chondritic' relative abundances. From this sample, an acid leached whole rock aliquot and two cpx separates were analysed. The first cpx separate was subjected to the acid leaching procedure used for KH-1 mineral separates. The second cpx aliquot was more harshly etched to investigate possible zoning which may be reflected in the isotopic composition, as well as to determine the effect on the acid treatment on possible differential leaching of ⁸⁷Sr.

The two samples were very friable and were gently crushed and sieved to a grain size of 0.1–0.3 mm. Clean grains without visible inclusions were separated by handpicking. For the opx separate of KH-1, only grains >2 mm were taken. These grains were then washed in warm 2M HCl for 20 min in an ultrasonic bath. After a 5-min treatment with 5% HF, the samples were crushed and a fraction between 100 and 200 mesh was taken for final handpicking under a binocular microscope. This separate was leached for 5 min in 5% HF and then again washed in warm 2M HCl, followed by an ultrasonic treatment in warm 2M HCl for 20 min.

The KH-1 whole rock leach was produced by leaching an aliquant (0.9779 g) of powdered (<200 mesh) bulk rock in 2M HCl for 20 min. The HCl solution was then filtered. Weight loss in this procedure (57.9 mg) is not representative of the actual amount of material which went into solution due to the loss of a very fine-grained fraction during filtering.

Two SC-1 cpx separates were prepared by the method described above. One cpx fraction (26.8 mg) was further treated for 45 min in concentrated HF and HClO₄ in an ultrasonic bath. After this etching, the sample was washed with high purity water. The etching solution plus the water wash were evaporated and processed as sample 'SC-1 cpx etch'. The remaining cpx (19.3 mg) was dissolved to become sample 'SC-1 cpx residue'. Weight loss during this procedure was 7.5 mg (28%) and is believed to have been completely retained in the etch solution.

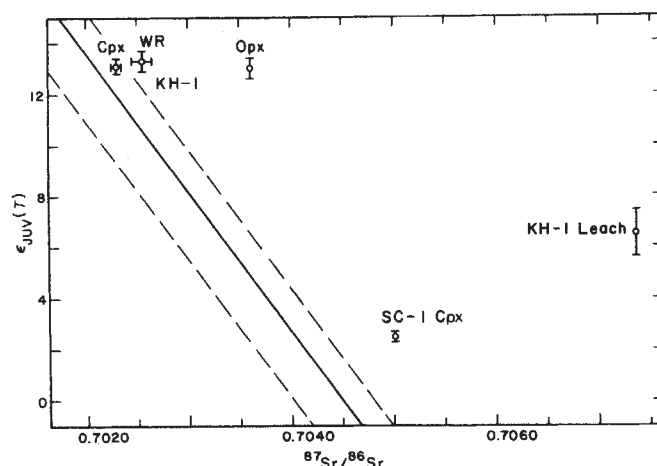


Fig. 1 $\epsilon_{\text{JUV}}(T)$ plotted against $^{87}\text{Sr}/^{86}\text{Sr}$. $\epsilon_{\text{JUV}}(T)$, expressed in parts per 10⁴, is the fractional deviation of $^{143}\text{Nd}/^{144}\text{Nd}$ in samples from that existing in a chondritic reference reservoir. The Sr–Nd trend line observed for young basaltic rocks and the approximate range of values (dashed lines) about this trend are adopted from O'Nions *et al.*¹⁶ and DePaolo and Wasserburg¹⁷. Note the observed Nd isotopic equilibration, but Sr disequilibrium in the minerals of KH-1 and the offset of the SC-1 cpx point to higher $^{87}\text{Sr}/^{86}\text{Sr}$ than found for the basalts.

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Table 1 Analytical results

Sample	Weight (mg)	[Sm] (p.p.m.)	[Nd] (p.p.m.)	$^{147}\text{Sm}/^{144}\text{Nd}^*$	$^{87}\text{Sr}/^{86}\text{Sr}^{\ddagger}$	$^{143}\text{Nd}/^{144}\text{Nd}^{\S}$	$\epsilon_{\text{JUV}}(T)^{\parallel}$
KH-1 cpx	107.6	1.42	2.71	0.3171	0.70229 ± 6	0.513308 ± 15	13.1 ± 0.3
KH-1 opx	730.7	0.0230	0.0249	0.5578	0.70361 ± 4	0.513301 ± 21	13.0 ± 0.4
KH-1 WR	299.1	0.242	0.456	0.3208	0.70255 ± 9	0.513319 ± 21	13.3 ± 0.4
KH-1 'Leach'				0.1918	0.70736 ± 3	0.512971 ± 48	6.5 ± 0.9
SC-1 cpx	56.3	3.66	10.3	0.2150	0.70501 ± 4	0.512764 ± 11	2.5 ± 0.2
SC 1 WR	327.7	0.680	1.89	0.2174		0.512763 ± 24	2.5 ± 0.5
'SC-1 cpx etch'	7.5				0.70488 ± 3		
'SC-1 cpx residue'	19.3				0.70498 ± 4		

Cpx, clinopyroxene; opx, orthopyroxene; WR, whole rock.

* Uncertainty ≈ 0.1%.

† Sr fractionation corrected to $^{86}\text{Sr}/^{88}\text{Sr} = 0.1194$; values measured for NBS 987 Sr show a total range of ±0.01% about $^{87}\text{Sr}/^{86}\text{Sr} = 0.71014$.

‡ Errors correspond to last digits and represent 95% C.L.

§ Nd measured as NdO^+ fractionation corrected to $^{148}\text{NdO}/^{144}\text{NdO} = 0.242436$ ($^{148}\text{Nd}/^{144}\text{Nd} = 0.241572$).

|| Ref. 15.

Procedures for dissolution, chemical separation, and isotopic analysis have been reported previously^{8,9}. Samples were totally spiked after dissolution for Sm and Nd concentration determination. Only the etched cpx separate and its leach from SC-1 were spiked for Sr concentration measurement. Blanks for Sm, Nd and Sr for the chemical procedures used were 8, 50 and 300 pg respectively.

Nd and Sr isotopic results and Sm and Nd concentrations are presented in Table 1. Distinct differences are apparent in the $^{143}\text{Nd}/^{144}\text{Nd}$ of samples SC-1 and KH-1. However, minerals within a single sample have Nd isotopic compositions which agree within analytical uncertainty. This is dramatically demonstrated in the case of KH-1 where a large spread in $^{147}\text{Sm}/^{144}\text{Nd}$ was measured for opx and cpx. On the other hand, despite the careful pretreatment of the samples, $^{87}\text{Sr}/^{86}\text{Sr}$ in KH-1 is considerably higher in opx than in cpx.

The observed equilibrium for Nd between cpx, opx (and the whole rock) of KH-1 demonstrates that Nd was in diffusive equilibrium at the time of the nodule's eruption on to the surface. These findings are similar to those reported by other workers¹⁶⁻¹⁸ who found cpx and garnet to be in Nd isotopic equilibrium in garnet lherzolite xenoliths at the time of their inclusion in South African kimberlites. On the other hand, in KH-1, the measured Sr fractions derived from the different mineral separates are not isotopically equilibrated.

Possible explanations for the observed differences in $^{87}\text{Sr}/^{86}\text{Sr}$ in KH-1 could be: (1) Sr diffusion in the mantle is much slower than that of Nd; (2) radiogenic growth of ^{87}Sr since the last time of Nd equilibration; (3) residual contamination of the measured mineral phases not removed by the leaching procedure; (4) (partial) equilibration with a fluid enriched in ^{87}Sr .

Although there is no direct experimental evidence for a difference between the diffusivity of Nd and Sr, some inferences could be drawn from the diffusive behaviour of M^{3+} and M^{2+} ions and the influence of ionic radii on diffusivity in silicate melts¹³. These data suggest the importance of charge in influencing diffusivity and imply that Nd should diffuse slower than Sr. The evidence above implies that, at least in the case of KH-1, the measured differences in $^{87}\text{Sr}/^{86}\text{Sr}$ were introduced after, or at the time of, the last equilibration of the Nd system. Because of the high precision obtained in the Nd isotopic measurements and the large spread in $^{147}\text{Sm}/^{144}\text{Nd}$ between opx and cpx, an upper limit of 8 Myr can be set for the time of the last Nd equilibration. If this also records the time of the last complete Sr equilibration, then taking the extreme limit of no ^{87}Rb decay in cpx ($^{87}\text{Rb}/^{86}\text{Sr} = 0$) an $^{87}\text{Rb}/^{86}\text{Sr} = 11.6$ would be required in opx to produce the measured differences in $^{87}\text{Sr}/^{86}\text{Sr}$ between the mineral separates within the 8 Myr upper limit imposed by the Nd equilibration. This ratio is 50–100 times the actual value measured for opx from other ultramafic nodules^{1,3-5}. Therefore, it seems very unlikely that the observed Sr disequilibrium is the result of *in situ* radiogenic growth of ^{87}Sr within the minerals.

Results for the surface leach of the KH-1 whole rock sample show the presence of an HCl soluble surface coating with $^{87}\text{Sr}/^{86}\text{Sr} = 0.70736$. Nd in this surface leach was very low in concentration ($<10^{-8}$ g Nd per g of bulk rock) with $^{143}\text{Nd}/^{144}\text{Nd} = 0.51297$ corresponding to $\epsilon_{\text{JUV}}(T) = +6.5$. Two sources for this radiogenic Sr component can be proposed: (1) surface contaminant deposited from the vapour phase of the host basalts in the Kilbourne Hole area. However, the observed $^{87}\text{Sr}/^{86}\text{Sr}$ in the leach is considerably higher than found for the basalts in the area¹; (2) deposition on to the minerals of salts contained in the groundwater of the area. This is supported by the similarity in $^{87}\text{Sr}/^{86}\text{Sr}$ measured for this surface wash and that found for caliche from the Rio Grande Rift (E. Padovani, personal communication). Therefore, it follows that the disequilibrium found for $^{87}\text{Sr}/^{86}\text{Sr}$ in the minerals from KH-1 may be due to incomplete removal of this surface contaminant, or possibly even a small degree of diffusion of this Sr contaminant into the mineral grains. The effect of this contaminant would be much more pronounced for minerals with low Sr content such as opx (and olivine), but would have little effect on cpx due to its high Sr content. On the other hand, the leaching experiment performed on SC-1 cpx does not indicate the presence of any residual contaminant after the initial acid washings. However, note that, due to the very high Sr concentration of SC-1 cpx (126 p.p.m.), this experiment may not be very sensitive towards detecting residual contamination. In addition, there is no evidence from this experiment to indicate preferential leaching of ^{87}Sr by the various acid treatments.

Although the exact cause of the observed mineral Sr isotopic disequilibrium is not tightly constrained by the data presented here, it is apparent from this and previous work¹⁰⁻¹² that Nd isotopic equilibration between major mantle minerals (cpx, opx, garnet) is achieved on time scales which are much shorter than predicted from experimental subsolidus diffusion data.

Figure 1 displays the isotopic results as $\epsilon_{\text{JUV}}(T)$ plotted against $^{87}\text{Sr}/^{86}\text{Sr}$. This figure and the major element data⁷ indicate that KH-1, with $\epsilon_{\text{JUV}}(T) = 13.1$, is a suitable parent material from which a magma similar in composition to mid-ocean ridge basalts could be derived. If KH-1 is representative of LREE-depleted primitive nodules in this area, then the results provide evidence for the existence of oceanic-type mantle underlying the Rio Grande Rift. A similar conclusion was reached by Reid and Woods¹⁴, based on a major element comparison for nodules from the same area with those found at Salt Lake Crater, Hawaii.

The Sm–Nd model ages of SC-1 and KH-1 (T_{ICE}) of 814 ± 157 and 819 ± 26 Myr, respectively, are in reasonably good agreement. One explanation for this concordance is that the source for these two samples was initially depleted in incompatible elements close to 800 Myr ago from a primordial reservoir with 'chondritic' relative REE abundances. If the group of 'primitive' nodules, of which KH-1 and SC-1 are only two examples, do represent mantle samples which have rather

uncomplicated differentiation histories, then these nodules can provide important constraints on the nature of the mantle. Of particular interest is that this group of nodules shows very similar major element compositions, yet has highly variable abundances of the incompatible elements. As all the radiometric systems also belong to the group of incompatible elements, the large variability in the degree of depletion of these elements in 'primitive' nodules such as KH-1 and SC-1 has profound implications for the interpretation of the isotopic data. In particular, the similar major element compositions of SC-1 and KH-1 clearly show that incompatible element mobilization can be accomplished without significant depletion in the basaltic component (that is, CaO , Al_2O_3). Therefore, the isotopic data presented here for KH-1 suggest that mantle with 'undepleted' abundances of CaO and Al_2O_3 can have Nd and Sr isotopic composition similar to that assumed for the 'depleted' oceanic mantle.

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Effects of biotic and abiotic elicitors on phytoalexin metabolism in soybean

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Elicitors induce accumulation of phytoalexins in plants¹. Recently Yoshikawa² concluded that biotic and abiotic elicitors act by different mechanisms. Thus a biotic elicitor, such as the cell walls of *Phytophthora megasperma* var. *sojae*, stimulated glyceollin synthesis in soybean cotyledons but had no significant effect on glyceollin degradation. In contrast, the abiotic elicitor HgCl_2 had only a slight effect on the biosynthetic activity but strongly inhibited glyceollin degradation. Here we report results which are contrary to Yoshikawa's conclusion.

Yoshikawa estimated biosynthetic activity by pulse-labelling with ^{14}C -L-phenylalanine. It is, however, not known whether uptake of this compound was influenced by elicitor treatment. For measurement of glyceollin degrading activity, cotyledons were incubated with glyceollin. The drawback of this method is that externally supplied glyceollin could be converted differently than the glyceollin in the internal pool. Because of the importance of elicitors in phytoalexin research¹, we have reinvestigated the effect of a biotic and an abiotic elicitor on glyceollin synthesis and conversion in soybean cotyledons by pulse-labelling with $^{14}\text{CO}_2$. By using high pressure liquid chromatography (HPLC) for separation of products, it was also possible to determine rate of synthesis and conversion of 3,6 α ,9-trihydroxypterocarpan, a precursor of glyceollin³.

Figure 1 shows the accumulation of glyceollin (containing the three isomers^{6,7}) and trihydroxypterocarpan (THP) after induction with the glucan from *P. megasperma*⁵ or HgCl_2 as elicitor. Induction with glucan results in somewhat faster and higher accumulation of both compounds compared to the induction with HgCl_2 . In the latter case glyceollin accumulation levels off earlier than THP accumulation. Controls with sterile cut cotyledons, treated only with buffer, contained only very small amounts of both compounds ($<1\text{ }\mu\text{g}$ per g fresh weight) which could not be quantitated.

The integrated curves for the rates of synthesis of glyceollin and THP after induction with either elicitor (Fig. 2) show no significant differences between the effects of the biotic and abiotic elicitor. In both cases the rate of THP synthesis is higher than that of glyceollin synthesis. Half-times of conversion were

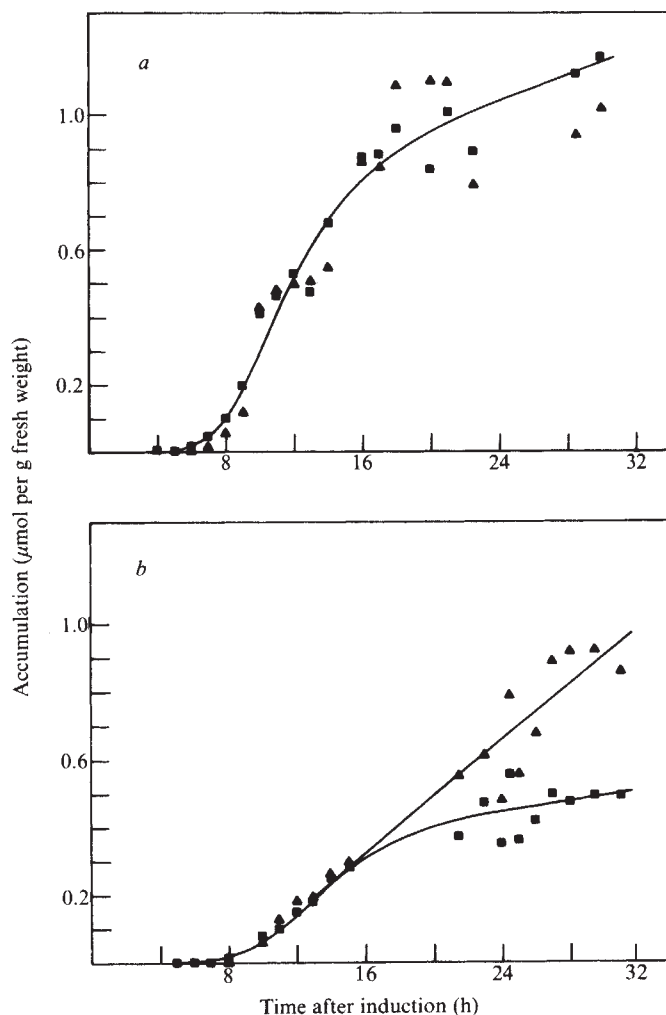


Fig. 1 Effect of glucan from *P. megasperma* and of mercuric chloride on accumulation of glyceollin (■) and trihydroxypterocarpan (▲) in soybean cotyledons. Detached cotyledons from 5-day-old seedlings of the soybean [*Glycine max* (L.) Merr.] cultivar Harosoy 63 were wounded as described by Ayers *et al.*⁴, except that wounds were made on the lower surface of the cotyledons. An aliquot (100 μl) of an elicitor solution (a, biotic elicitor: heat-solubilized mycelial wall of *P. megasperma* var. *sojae*⁵ (glucan), 300 μg glucose-equivalents ml^{-1} ; b, abiotic elicitor: 1.5 mM HgCl_2) was placed on wounded areas of each of 20 cotyledons, and these were incubated on moist filter paper in Petri dishes at 30 °C in the dark. Both elicitors were used in concentrations optimal for glyceollin accumulation. At the times indicated, the cotyledons were extracted with 100 ml of 60% ethanol by vacuum infiltration for 30 min, then the ethanol was removed under reduced pressure, the remaining solution was extracted with 100 ml of ethylacetate and the organic phase dried over Na_2SO_4 . After removing the solvent under reduced pressure, each sample was chromatographed on Sephadex LH-20 (30 $\times$ 2.5 cm) in methanol. Separation of glyceollin, 3,6 α ,9-trihydroxypterocarpan and minor isoflavonoid components was then achieved by HPLC as described by Zähringer *et al.*³. The pterocarpan components were quantitated with a Waters Modul 730 using an extinction coefficient of 10,000 (ref. 4).

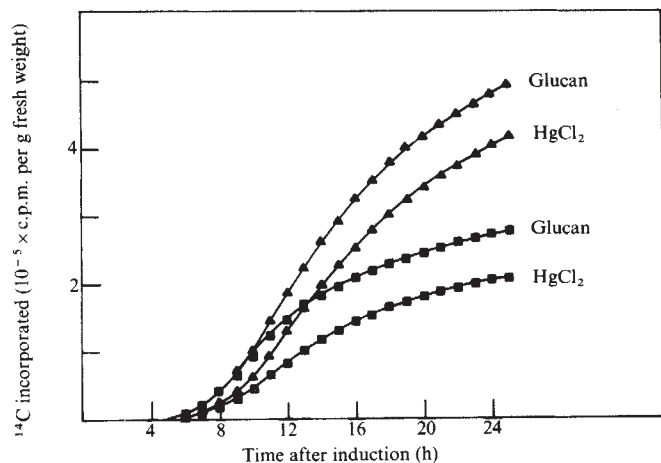


Fig. 2 Synthetic activity for glyceollin (■) and trihydroxyperocarpan (▲) production after treatment with glucan or mercuric chloride. Each set of 20 cotyledons was incubated in a 800-ml capacity gas-tight chamber with 100 μ Ci 14 CO₂ (53.5 mCi mmol⁻¹) under illumination (Osram L 40 W/25, 9,500 lx) for 1 h before sampling. Radioactivity of the components was determined in the efflux of HPLC column with a Beckman Model LS-335 scintillation spectrometer using a toluene scintillation fluid. Radioactivity of glyceollin and THP (in c.p.m. per g fresh weight) was plotted against time after induction. For better comparison with the data obtained for accumulation, the curves were graphically integrated. Estimation of degradation activity (data not shown): 20 Petri dishes, each with 20 cotyledons, were transferred 8 h after induction with the elicitors into a 35 l chamber and were incubated under illumination with 1 mCi 14 CO₂ (53.5 mCi mmol⁻¹) for 5 h. The pulse was followed by a chase phase (with illumination), in which 14 CO₂ was replaced by air blown through the chamber. Twenty samples were analysed between 20 and 50 h after induction for radioactivity as described above.

obtained from pulse-chase experiments. Straight lines on semilogarithmic plots were obtained by linear regression analysis from which the following half-times were estimated: glyceollin (glucan) 112 $\pm$ 63 h; glyceollin (HgCl₂) 89 $\pm$ 53 h; THP (glucan) 39 $\pm$ 8 h; THP (HgCl₂) 36 $\pm$ 13 h. Determination of half-times in the controls was not possible due to the very low amounts of glyceollin and THP.

These results are not in agreement with Yoshikawa's conclusion² that biotic and abiotic elicitors have a diverse mode of action, at least in soybean cotyledons. Rates of synthesis of glyceollin and of its precursor trihydroxyperocarpan, as well as rates of conversion, are very similar with the two types of elicitors. From the relatively long half-time of conversion of glyceollin, one can conclude that accumulation of glyceollin isomers results mainly, if not exclusively, from stimulation of their synthesis by the elicitors. The rate of conversion of THP very probably represents its transformation to the 2-prenyl derivative, a potential precursor of glyceollin³.

Our results are in agreement with the suggestion of Hargreaves⁸ that mercuric chloride causes the production or release of constitutive phytoalexin elicitors. Such elicitors would be expected to have the same mode of action as externally supplied biotic elicitors.

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Precocenes induce effect of juvenile hormone excess in *Locusta migratoria*

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Precocene I and II (PI and PII)¹ and synthetic analogues²⁻⁴ induce symptoms of permanent juvenile hormone (JH) deficiency in certain hemipteroid^{1,5,6} and orthopteroid⁷⁻¹¹ insects. Younger nymphs treated with precocenes moult to precocious prothetelic nymph-adult intermediates, termed adultiforms⁸, whereas treatment of older nymphs (too late for obtaining morphogenetic response) or of adults results in lack of oocyte development. The precocenes act as 'anti-allatins', causing atrophy of the corpora allata (CA)^{8,12-14}. Recent findings suggest that the CA specifically metabolize precocenes to cytotoxic 3,4-epoxy-precocenes, possibly due to the epoxidases present therein for JH biosynthesis; the accumulating precocene-epoxide seems to poison the glands^{15,16}. This theory is supported by the claim that active CA are required for the effect of PII in *Oncopeltus*^{6,17}. We demonstrate here that 7-ethoxy-PII ('Precocene III' = PIII), or PII itself, applied to last instar *Locusta* nymphs, induce an effect strictly typical of that of JH excess, in spite of eventually exerting the conventional anti-allatin effect. Our findings provide strong circumstantial evidence that supposedly inactive CA of these nymphs are temporarily activated by the precocenes, resulting in an outburst of JH biosynthesis before the glands are inhibited or destroyed.

Crowded nymphs of *Locusta migratoria migratorioides*, kept in routine conditions used in our laboratory⁸, were used for the experiments. PIII (ZR 3623, Zoecon) topically applied in acetone to 0-27-h-old fifth (last) instar nymphs caused a portion of them to moult to metathetelic adultoids, that is, to sixth (supernumerary) instar nymphs with some adult characteristics, or to sixth instar nymph-adult intermediates (Fig. 1, AD). These adultoids were morphologically similar to those obtained after implantation of extra CA into early fifth instar nymphs^{18,19}. Some adultoids even exhibited an extra (sixth) moult, although they died in its course, being unable to shed off the exuvium. Another portion of the fifth instar nymphs treated moulted to 'imperfect adults' (Joly's¹⁸ 'imago imparfait'). These are adults with curled or twisted wings which occasionally (5-10%) also occur among normal locusts (controls, C in Fig. 2), usually due to external disturbances during adult emergence and/or expansion of the wings. Imperfect adults are obtained, however, much more frequently after implantation of extra CA, presumably mostly due to internal disturbances; their presence in relatively large numbers indicates a slight metathetelic effect¹⁸. The third portion of the nymphs moulted to morphogenetically normal adults.

With increasing doses of PIII the number of metathetelic individuals increased (Fig. 2), up to a maximum (10 adultoids and 6 imperfect adults out of 23 nymphs treated) at 50 μ g per nymph. Further increase of the dose, however, resulted in a decrease of the metathetelic forms. At 500 μ g per nymph metathetely disappeared; with regard to morphogenesis (but not for other effects, see below) the results were similar to those obtained in acetone treated controls. Were PIII a JH analogue, increasing doses would have eventually resulted in 100% adultoids. As a reverse trend was observed, PIII would not seem to act as a JH analogue in spite of inducing the effect of JH excess at lower doses.

The duration of the fifth instar was not distinctly affected by the dose when subsequent adultoids or imperfect adults were obtained; the respective averages for these two categories were

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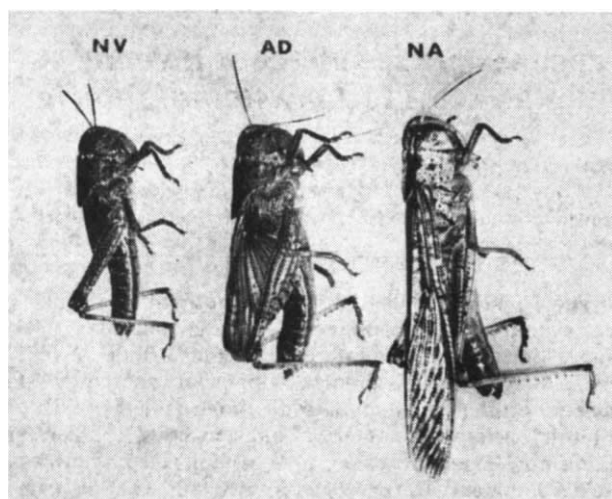


Fig. 1 Sixth instar metathetetic adultoid (AD) of *Locusta migratoria* obtained after topical application of 7-ethoxy-precocene II ('precocene III') to 0–27-h-old fifth (last) instar nymphs, in comparison with normal acetone-treated fifth instar nymph (NV) and normal (sixth instar) adult (NA).

189 (range 122–242) h and 228 (122–315) h. For morphogenetically normal adults the overall value was 245 (187–357) h, but here some dose-dependent trend was observed; with increasing doses the average duration of the fifth instar increased from 220 (187–286) h (25 or 50 μg PIII per nymph) to 275 (215–357) h (500 μg PIII). Morphogenetically normal acetone-treated controls gave 258 (215–311) h. Metathetely seems to be correlated, therefore, with shorter duration of the previous instar.

The same locusts from which Fig. 2 was obtained were maintained for 23–37 days after moult to adults or adultoids. The colour of all survivors was then recorded and the locusts were dissected; the state of the oocytes was noted in females and the CA were examined in both sexes. Lack of vitellogenetic oocytes, absence of yellow colour and atrophied CA indicated an anti-allatin effect (see ref. 8). Vitellogenetic oocytes and

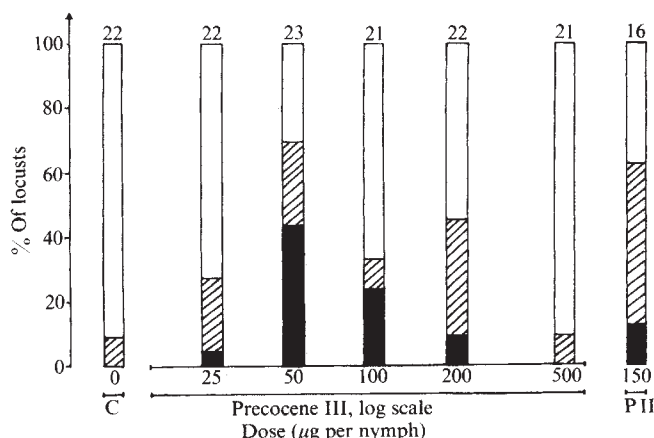


Fig. 2 Dose-response of precocene induced metathetely. 'C', acetone-treated controls; 'Precocene III' treatment with 7-ethoxy-precocene II; 'PII' treatment with precocene II. Each column represents a single experimental group. Number of locusts in each group after the next (from fifth to sixth instar) moult (constituting 100% for the ordinate) is noted on the top of each column. Mortality during the fifth instar did not exceed two locusts in any group. Filled area sixth instar adultoids; hatched area; sixth instar imperfect adults; unshaded area; sixth instar morphogenetically normal adults.

presence of yellow colour were sufficient for recognizing functional CA in imperfect or morphogenetically normal adults. Adultoids, however, especially with dominating larval features, often did not show signs of sexual maturation (see also ref. 18); in these cases conclusions were limited to the results of the examination of the CA. Dose-response data (Fig. 3) yielded the customary sigmoid curve, revealing that PIII did induce the conventional anti-allatin effect of the precocenes, in spite of exerting the metathetelic effect. Interestingly, 50 μg PIII per nymph, the dose which induced maximum metathetely (Fig. 2), was the closest to ED_{50} (50% of locusts with non-functional CA) for the anti-allatin effect (Fig. 3).

A single experiment with PII (Calbiochem) revealed that the double effect, induction of metathetely (PII, Fig. 2) and anti-allatin action (PII, Fig. 3), is not confined to PIII. Studies of dose-response relationships of PII with regard to both effects are under way.

Only JH or bioanalogues are known to induce metathetely. Since precocenes do not seem to act as JH analogues (see above), the metathetely observed by us is presumably a result of

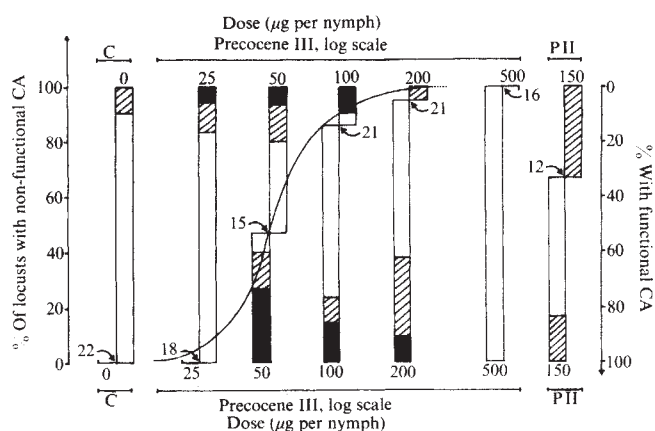


Fig. 3 Dose-response relationships of the anti-allatin effect of precocenes. Number of locusts at the examination of the anti-allatin effect (23–37 days after moult to sixth instar adultoids or adults) is noted with arrows at the meeting point of rising and hanging columns for the same dose and substance. Locusts were the same as used for Fig. 2, except for some which died (naturally, accidentally or during extra moult of some adultoids) or were lost between examination of metathetely (Fig. 2) and of anti-allatin effect. The sigmoid curve is the dose-response curve for the anti-allatin effect of 7-ethoxy-precocene II ('Precocene III'). Abbreviations and shading as in Fig. 2.

endogenous JH excess. The CA are the only known source of endogenous JH in *Locusta* and JH titre in the haemolymph is undetectable or very low in early last instar nymphs^{20–22}. Thus, in accord with conventional concepts, *in situ* CA seem to be rather inactive at this time (although transplanted necessarily denervated CA may show activity, see refs 20, 23). At present the sole feasible explanation for metathetely seems to be, therefore, that PIII (and PII) directly or indirectly activates the nymphs' own CA resulting in an outburst of JH biosynthesis and release. Activated CA, however, presumably rapidly metabolize the precocenes to cytotoxic precocene-epoxides which then inhibit or destroy the glands^{15,16}. Thus, precocene-induced CA activation is probably very temporary. More active CA may destroy themselves more quickly, leaving less time for JH biosynthesis. If so, with increasing doses the balance would be gradually shifted to produce less JH and more precocene-epoxide, resulting in decrease of metathetely and increase of anti-allatin effect. This reasoning exactly fits our results, including the eventual complete loss of the effect of JH excess at high doses.

None of the adultoids or adults turned green in the present experiments. Green colour is caused by JH in *Locusta* nymphs^{18,19,23}, but its absence can easily be explained if precocene-induced CA activation is indeed temporary. The timing of susceptibility of the integument to JH may differ for morphogenetic and for colour effects, or alternatively, a longer lasting or continuous JH titre (but a low one, as normal solitary nymphs are mostly green without morphogenetic disturbances) may be needed for green colour induction. Both explanations can be reconciled with previous experimental findings and theoretical considerations^{18,21}. If this is so, precocene-induced temporary activation of the CA may occur in other stages too, but it may not become overt. In younger instars no metathetely would be discernible and a short outburst of JH excess may be just as insufficient for inducing green colour as in last instar nymphs. Moreover, if our interpretation of the results is correct (on the basis of present knowledge of insect endocrinology no other explanation can be offered without wild speculation), differences in susceptibility of different species, or of different instars of the same species, differences in effectiveness of different methods of administration (such as topical application and residual coating methods) and eventually possible development of precocenes to insect control agents, may all be related to precocene-induced activation of the CA.

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Killed *Listeria monocytogenes* vaccine is protective in C3H/HeJ mice without addition of adjuvants

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Resistance of mice to the facultative intracellular parasite *Listeria monocytogenes* involves cellular immunity¹, as it can be transferred passively with viable lymphoid cells² but not with serum. Induction of protective immunity can only be achieved by vaccination with sublethal numbers of viable listeria^{3,4} or with dead listeria in combination with certain adjuvants^{5,6}. Killed listeria vaccines as such are not protective. Effective adjuvants are lipopolysaccharide (LPS)⁵, dextran sulphate (molecular weight 500,000) and suramin⁶, whereas Freund's complete adjuvant is ineffective⁶. All these polyanionic substances have a

shortlived deleterious effect on macrophage function⁵⁻⁷. It seems necessary for their adjuvant activity that they be present during the induction of immunity, most probably resulting in incomplete degradation of the dead listeria within the macrophage. During the effector phase, however, the adjuvants need to be virtually absent^{8,9}, so that macrophages are unhampered in eliminating the infective bacteria of an eliciting listeria injection. We describe here our finding that LPS-nonresponder C3H/HeJ mice, unlike H-2 identical C3HeB/FeJ or H-2 unrelated mice, can develop resistance to a lethal dose of viable *L. monocytogenes* on a single injection with dead listeria vaccine without any adjuvant added. This suggests that the C3H/HeJ mouse has some kind of 'intrinsic adjuvant'. If present, such an intrinsic adjuvant would, in analogy to the effective adjuvants⁵⁻⁷, probably be a polyanionic substance. By means of protamine precipitation¹⁰, we have found evidence for the presence of such a substance in the serum of C3H/HeJ mice.

C3H/HeJ (H-2^k), C3HeB/FeJ (H-2^k) and BALB/c (H-2^d) mice, aged 10-14 weeks, received 10⁸ heat-killed listeria or a control saline injection. The dose of 10⁸ listeria was shown to be optimally protective if injected in combination with adjuvant⁶. Seven days later, protective immunity was tested by injection of 20 × LD₅₀ of live listeria and subsequent daily inspection of the animals for two weeks (LD₅₀ = the dose resulting in 50% death of unimmunized BALB/c mice in similar conditions). None of the saline-injected mice, except for one C3H/HeJ mouse, survived the challenge with viable listeria (Table 1). Vaccinated C3H/HeJ mice, however, in contrast to BALB/c and even to H-2 identical C3HeB/FeJ mice, were significantly protected against the lethal challenge.

In view of the current ideas about the development of resistance towards intracellular parasites^{6,7}, our results suggest the C3H/HeJ mouse to bear a kind of intrinsic adjuvant. This adjuvant might be cell-linked, perhaps identical to the macrophage defect¹¹. According to this hypothesis, defective macrophage processing would result in incomplete degradation of immunogenic elements of the dead vaccine, promoting the induction of protective immunity. During the effector phase, however, the macrophage defect of the C3H/HeJ mouse could be corrected by lymphokine production¹². Another explanation of our findings might be that the intrinsic adjuvant of the C3H/HeJ mouse is not cell (macrophage) linked, but that it is, for instance, a soluble serum factor. As the effective adjuvants are polyanionic substances, the serum of C3H/HeJ mice was investigated for such a substance by protamine sulphate precipitation¹⁰, and the C3H/HeJ mouse was compared with the C3HeB/FeJ and the BALB/c mouse. As Table 2 shows, a 'protamine precipitable factor' (PPF) was indeed present in C3H/HeJ serum. PPF was also found in C3HeB/FeJ and BALB/c serum, but in substantially lower concentrations.

It is possible that there is no relationship between the relatively high PPF level, the macrophage defect and other errors of the C3H/HeJ mouse, such as a defect in the early classical complement system¹⁴, most probably residing in the C_{3b} or C_{4b} inactivator system¹⁵. An interesting alternative explanation is, however, that there is a relationship or even identity between the abnormalities of the C3H/HeJ mouse. In that construction,

Table 1 Resistance of the mouse strains to a lethal listeria infection after injection with a dead vaccine

Mouse (sub)strain	Injection	
	Vaccine	Saline
C3H/HeJ	24/30	1/30
C3HeB/FeJ	2/30	0/30
BALB/c	0/30	0/30

Groups of five male C3H/HeJ, C3HeB/FeJ and BALB/c mice were injected intraperitoneally with 10⁸ heat-killed (60 min, 56 °C) listeria or saline. Seven days later, the animals were challenged intraperitoneally with live (20 × LD₅₀) listeria. The numbers of survivors on the numbers of mice challenged were recorded for 2 weeks. Results of six such experiments are summarized above.

Table 2 Serum levels of protamin precipitable factor

Mouse (sub)strains	PPF serum level (units ml ⁻¹)	P value (Student's <i>t</i> -test)
C3H/HeJ	291 ± 32	
C3HeB/FeJ	133 ± 20	<0.0005 (two-sided)
BALB/c	68 ± 4	<0.0001

Groups of 20 male C3H/HeJ, C3HeB/FeJ and BALB/c mice were bled for individual sera by retro-orbital puncture at 11.30 p.m. To 20 µl of serial dilutions of these sera, 2 ml Veronal-buffered saline, pH 7.4, containing 0.15 mM Ca²⁺ and 0.5 mM Mg²⁺ (VSB⁺⁺)¹³ and 250 µg protamin sulphate, dissolved in 25 µl of VSB⁺⁺, were added. The mixture was incubated at room temperature for 20 min, whereafter the turbidity was determined by measuring the E₄₅₀ against a serum blanco. As found for heparin, which was used as reference, a linear relationship between the serum concentration and the turbidity was observed. PPF concentrations have been expressed in equivalents of heparin units.

PPF would resemble heparin in more than one respect: heparin is easily precipitated by protamin, it is an inhibitor of several enzyme activities¹⁶, most probably also within the macrophage into which it is easily incorporated¹⁷. Moreover, it mimics the effects of the C_{3b} inactivator system in preventing C_{3b}-B complex formation¹⁸. The identity and activities of PPF will be subject of further study.

Whatever the case, our observations on the development of protective immunity on injection with a dead listeria vaccine and on the high PPF levels in the C3H/HeJ mouse are expected to bring new insights in both the acquisition of resistance to intracellular parasites and the defect(s) of the C3H/HeJ mouse.

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Mechanism of origin of complete hydatidiform moles

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Complete or 'true' hydatidiform mole, an abnormality of human gestation, is characterized by hydropic degeneration of all placental villi, marked hypertrophy of the trophoblast, absence of a fetus and a propensity to become malignant. The chromosome constitution of complete moles is usually 46,XX^{1,2}, and Kajii *et al.* reported that the entire genome in seven out of seven cases was paternal in origin, with all centromere markers homozygous for paternal heteromorphisms³. These observations, since confirmed^{4,5}, can be explained by the fertilization of an 'empty' egg—no effective genome—by either a haploid sperm that then duplicates without cytokinesis, to restore the diploid number, or by a diploid sperm resulting from failure of the second meiotic division. We report here a study of a series of complete moles that shows the first alternative to be correct in the majority of cases.

Diploidization of the sperm would result in an entirely homozygous conceptus, while fertilization with a diploid sperm would give rise to heterozygosity for some of those genes heterozygous in the father and situated distal to the points of exchange. As all chromosome heteromorphisms are situated at or very near the centromere they cannot be used alone to distinguish between the two mechanisms of origin. We therefore studied the phosphoglucomutase₁ (PGM₁) genotypes, as well as both C and Q-band chromosome heteromorphisms of an unselected series of complete moles and their parents from Baltimore and Hawaii.

PGM₁ has two common alleles with gene frequencies of approximately 0.76 and 0.24 (ref. 6). The locus is on the short arm of chromosome 1 and maximum likelihood estimates suggest that in males it is 50 centimorgans from the centromeric heteromorphic band at q12 (ref. 7). We restricted our analyses

of non-centromeric markers to PGM₁ because that is the only locus available to us (1) with a genotype that can be determined reliably in both parental blood and fetal tissue; (2) with a high frequency of parental heterozygosity; and (3) which is situated sufficiently far from the centromere to ensure frequent recombination with it.

Results for 24 moles are shown in Table 1. In 20 cases successful chromosome studies were completed on cells cultured from the mole and both parents, and in a further 4 from mole and mother alone. Twenty-one moles had a 46,XX constitution, one had an additional medium-sized chromosome in the few cells we could analyse, one had a variable count with a mode at 85,XXXX and one was 46,XY. Cultures from nine 46,XX moles were treated with 5-bromodeoxyuridine (BUdR) and the cells were stained with acridine-orange to investigate X-chromosome allocycly⁸. All nine moles had a single allocyclic X in more than 96% of metaphases examined and were thus indistinguishable from cells from normal females.

In 22 moles the centromeric heteromorphisms in all informative chromosomes appeared homozygous and clearly of paternal origin. The PGM₁ genotype of the mole and both parents was determined for 14 of these 22 moles and in a further 3 cases for the mole alone. In all 17 cases, including 6 in which the father was known to be heterozygous, the mole was homozygous. Based on gene frequencies, it can be estimated that, if the moles originated from a diploid sperm resulting from failure of the second meiotic division, 6.2 of the 17 tested moles would be heterozygous at the PGM₁ locus as would 4.0 of the 6 in which the father is known to be heterozygous. The absence of heterozygosity at the PGM₁ locus is significant both for the total number of moles tested ($P < 0.001$) and for the offspring of known heterozygous fathers ($P < 0.01$) and is therefore strongly supportive of a haploid sperm origin for most of the 46,XX moles.

Yamashita *et al.*⁹ reported the HLA specificities of 13 moles and their parents and in 11 informative matings the moles showed no heterozygosity. However, the maximum likelihood estimate for the distance of the HLA loci from the centromere of chromosome 6 in males is only 8 centimorgans (B. J. B. Keats, personal communication). Thus HLA is an inefficient locus for determining the exact origin of 46,XX moles and in a series of 11 the expectation of heterozygosity, given an origin by failure of meiosis II leading to a diploid sperm, is only 1.8, not significantly different from the zero observed. Lawler *et al.*¹⁰ tested five different loci in parents and moles and found the mole to be

Table 1 Cytogenetic and PGM₁ observations on 24 complete moles

No.	Chromosome constitution	Chromosome heteromorphisms*											PGM ₁ ⁺ type
		Chromosome no.											
			1	3	4	9	13	14	15	16	21	22	
K456	46,XX	Pat		aa	aa		ab		ab			ab	
		Mat	—	bc	ab	—	cd	—	bb	—	—	cc	—
		Mole		aa	aa		aa		aa			aa	
K487	46,XX	Pat	—								—	—	
		Mat	ab	—	—	—	—	—	—	—	aa	ab	—
		Mole	cc								bb	cc	
K513	46,XX	Pat		ab		ab	ab			aa			
		Mat	—	ac	—	aa	cd	—	—	ab	—	—	—
		Mole		bb		bb	aa			aa			
K515	85,XXXX	Pat		aa									
		Mat	—	bb	—	—	—	—	—	—	—	—	—
		Mole		aaaa									
K739	46,XX	Pat		ab		ab	ab	aa	aa				
		Mat	—	cc	—	ac	cd	ab	bb	—	—	—	—
		Mole		ac		bc	ac	ab	ab				
K901	46,XX	Pat	aa	ab		aa	ab	aa	ab	aa	ab	aa	1-1
		Mat	bb	cd	—	ab	ac	ab	ab	bb	ac	ab	1-1
		Mole	aa	aa		aa	bb	aa	aa	aa	bb	aa	1-1
K1040	46,XX	Pat	aa				ab		ab	ab	ab	ab	1-1
		Mat	bc	—	—	—	cd	—	aa	aa	ac	—	1-2
		Mole	aa				aa		aa	aa	bb	—	1-1
K1064	46,XX	Pat	—	—		—	—	—	—	—	—	—	—
		Mat	aa	ab	—	aa	—	—	ab	—	—	ab	—
		Mole	bb	cc		bb			cc			aa	1-1
K1200	46,XX	Pat		—			—	—	—		—	—	—
		Mat	—	ab	—	—	ab	ab	—	—	aa	ab	1-2
		Mole		aa			aa	aa			bb	cc	1-1
K1248	46,XX	Pat		ab			ab	aa	ab		ab	ab	1-2
		Mat	—	ac	—	—	ac	ab	bb	—	ac	ab	1-1
		Mole		aa			aa	aa	aa		aa	aa	1-1
K1250	47,XX,+C	Pat			ab		ab		ab				1-1
		Mat	—	—	bb	—	cd	—	cd	—	—	—	1-2
		Mole			aa		aa		aa				1-1
K1359	46,XX	Pat		aa	aa		ab				ab	aa	1-1
		Mat	—	ab	bc	—	bc	—	—	—	ab	ab	1-2
		Mole		aa	aa		aa				aa	aa	1-1
S22	46,XX	Pat	ab		ab		ab		aa	ab	ab	ab	
		Mat	aa	—	aa	—	aa	—	bc	cc	aa	bb	—
		Mole	aa		aa		aa		aa	aa	aa	aa	
S26	46,XX	Pat		ab	ab		ab	ab	aa	ab		ab	1-1
		Mat	—	bc	ab	—	bc	cc	ab	ac	—	ab	2-2
		Mole		aa	aa		aa	aa	aa	aa		aa	1-1
S28	46,XX	Pat		ab			ab	ab	aa		aa	ab	1-2
		Mat	—	ac	—	—	cd	ac	ab	—	ab	ac	1-1
		Mole		aa			aa	aa	aa		aa	bb	2-2
S30	46,XX	Pat		ab			ab	ab	ab		ab		1-2
		Mat	—	ac	—	—	ac	ac	aa	—	aa		1-1
		Mole		bb			bb	bb	aa		aa		1-1
S31	46,XX	Pat		ab			ab	ab	ab		ab	aa	1-1
		Mat	—	ac	—	—	ac	aa	aa	—	ab	bc	1-2
		Mole		aa			aa	bb	bb		aa	aa	1-1
S32	46,XX	Pat		aa				ab				ab	1-2
		Mat	—	ab	—	—	—	aa	—	—	—	ac	1-1
		Mole		aa				bb				aa	2-2
S35	46,XX	Pat		—			—	—	—		—	—	—
		Mat	—	ab	—	—	ab	—	ab	—	ab	ab	2-2
		Mole		cc			cc		cc		cc	cc	1-1
S39	46,XX	Pat		ab			ab		ab		ab	aa	1-2
		Mat	—	ac	—	—	ac	—	cd	—	aa	ab	1-2
		Mole		aa			aa		aa		aa	aa	2-2
S40	46,XX	Pat		ab	ab		ab		ab		ab	ab	1-1
		Mat	—	ac	ab	—	bc	—	cc	—	aa	aa	1-2
		Mole		bb	aa		aa		aa		aa	aa	1-1
S41	46,XY	Pat	ab				ab			aa		ab	1-2
		Mat	aa	—	—	—	ab			ab		ab	1-1
		Mole	ab				aa			aa		aa	1-1
S45	46,XX	Pat		aa			aa	ab	ab			ab	1-1
		Mat	—	bb	—	—	ab	cc	bb	—	—	ac	1-1
		Mole		aa			aa	aa	bb			aa	1-1
S46	46,XX	Pat		aa			ab	ab	ab		aa	ab	1-2
		Mat	—	ab	—	—	cd	aa	cc	—	ab	ab	1-2
		Mole		aa			aa	aa	aa		aa	ag	2-2

* Heteromorphisms of chromosomes 3, 4, 13, 14, 15, 21 and 22 were studied on Q-banded preparations¹² while those of chromosomes 1, 9 and 16 were studied on C-banded slides¹³; a, b and c are symbols and do not represent specific heteromorphisms.

† PGM₁ genotyping was carried out on parental red cells, molar tissue and cultured fibroblasts by cellulose acetate electrophoresis¹⁴. In each case, the result from molar tissue assay was confirmed in cultured cells derived from the specimen.

homozygous in 18 informative tests in which the father was heterozygous. However the distribution of the 18 tests among the loci examined is not given and, as the centromere distance is not shown for many of the loci, it is not possible to determine the significance of their data, although they support a haploid origin.

Two atypical moles in our series are worthy of comment. In one, a 46,XX mole (K739), many of the centromeric heteromorphisms appeared heterozygous and comparison with the parental chromosomes showed this mole to be the product of a normal conception containing both a paternal and a maternal genome. We could not determine the origin of the single 46,XY mole (S41) because the centromeric heteromorphisms and PGM₁ observations were compatible with either a normal conception or fusion of two sperm with an empty egg, although they excluded fertilization by a diploid sperm. Observations on a 46,XY mole have been reported that suggest a paternal origin resulting from the fertilization of an empty egg by either two haploid sperm or a diploid sperm that arose by failure of the first meiotic division¹¹.

The observation that there are at least three mechanisms of origin for complete moles, each with different genetic implications, is important in the formulation of a hypothesis to explain the propensity of complete moles to undergo malignant transformation. It would be interesting to know whether such a propensity is a property of all three types of complete moles or whether it is restricted to, or more prevalent in, any one class.

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Bone-forming cell line derived from embryonal carcinoma cells

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The use of established cell lines of various types has provided information on the properties of certain stem cells and on the conditions of their differentiation. No bone-forming cell line has so far been described. We report here the isolation, from *in vitro* differentiating teratocarcinoma cells¹, of a line which, on subcutaneous injection into syngeneic mice, gives rise to ossicles. The appearance of ossicles is preceded by the formation of a cartilaginous matrix, thus reproducing what has been described as endochondral differentiation². The ossicles obtained are rapidly colonized by host marrow, are non-malignant and stop growing 2 weeks after injection of the cells. In certain conditions, however, osteosarcomas, chondroosteosarcomas and fibrosarcomas can be obtained. From these tumours, cell lines have been isolated which retain *in vitro* the properties of the cells found in the tumour.

Cell line 3/A/1D-1 was isolated from differentiated cultures of the embryonal carcinoma line PCC3/A/1 after *in vitro* differentiation¹. These cells have a pseudo-epithelial appearance, are contact-inhibited and aligned at confluency. In electron micrographs, numerous intracisternal virus-like A particles, but no C particles, can be seen.

Two weeks after subcutaneous injection of 2×10^6 cells into 129/Sv mice, an ossicle is formed at the injection site in 50% (45/91) of the mice. The formation of these tumours has been followed by histological examination (Fig. 1a–f). The initial step seems to correspond to the development of primitive mesenchymal cells (Fig. 1a) which, after 2 weeks, give rise to chondroblasts and chondrocytes (Fig. 1b,c). These cells show the typical transformation that is observed in endochondral ossification, with mineral deposits near the surface of the hypertrophied chondrocytes and in the peripheral matrix (Fig. 1c,d). Then, foci of haematopoiesis and typical marrow appear inside the bone cavity (Fig. 1e,f). Marrow smears show classical bone marrow cells, including erythrocytic and granulocytic cells as well as megakaryocytes. The ossicles thus obtained are benign tumours; they stop growing 2–3 weeks after injection of the cells and some ossicles have been observed for 1 yr without further changes. However, within 2–4 months, fibrosarcomas frequently appear (20 out of 42 129/Sv mice observed during 6 months). These tumours develop independently of whether or not the mice form ossicles. Thus, between the 20 mice presenting fibrosarcomas, only 10 had previously developed an ossicle. Three independent fibrosarcomas were transplanted 5 to 10 times in 129/Sv. In some of these tumours, bone cells could be observed.

The manner in which the cells were injected was of importance for further development of the tumours. When subcutaneous lesions were provoked by scratching, the percentage of ossicles formed increased from 50 to 100% (41/41). Furthermore, in most cases malignant osteosarcomas and/or fibroosteosarcomas developed (Fig. 1g,h). In certain species, such as rabbit and rat, various treatments^{3,4} or inert substances^{5,6} are known to cause subcutaneous development of ossicles. Therefore the origin of the different elements found in ossicles formed upon injection of 3/A/1 D-1 cells was studied. First, no ossicle was ever observed in control 129/Sv mice injected with medium alone, with or without subcutaneous lesions (0/18). Therefore, the mere production of lesions is not sufficient to induce ossicle formation in mice. Second, mice from strains other than 129/Sv were injected with 3/A/1 D-1 cells. Whatever their major histocompatibility (H-2) haplotype, none developed any ossicles (0 out of 42 allogeneic injections). Even mice bearing an H-2^b haplotype (as do 129/Sv) were negative. The injection and the presence of the cells for only a few days (before allogeneic rejection) are insufficient for the development of ossicles.

To reduce the possibilities of a contribution by the host, 3/A/1 D-1 cells were injected into the eye of 129/Sv mice. In every case, the development of bone tissue could be observed. As in the case of subcutaneous injections, the process began by the appearance of mesenchymal cells, rapidly followed by chondrocytes and osteoblast cells. However, no bone marrow could be observed in these conditions.

A direct study of the origin of the different elements of the ossicles was then carried out. After injection of 3/A/1 D-1 cells into (129/Sv $\times$ C57BL)F₁ mice, marrow-containing ossicles were formed at the site of injection. The origin of the marrow cells present in such H-2^b/H-2^k hybrids was studied by cytotoxicity tests with anti-H-2 sera. Bone marrow cells were found to respond to both anti-H-2.2 (which detects private specificity 2 of H-2^b) and anti-H-2.32 (which detects private specificity 32 of H-2^k) sera (Fig. 2). Thus, the majority of bone marrow cells appear to be of host origin. To study stromal cells^{8,9}, 3/A/1 D-1 cells were injected into (129/Sv $\times$ Rb 163)F₁ mice which carry a metacentric chromosome (9-19) as marker. The bone cavities of the ossicles obtained were flushed and the cells put in culture. Karyotype analysis of epithelial and pseudofibroblastic cells 18

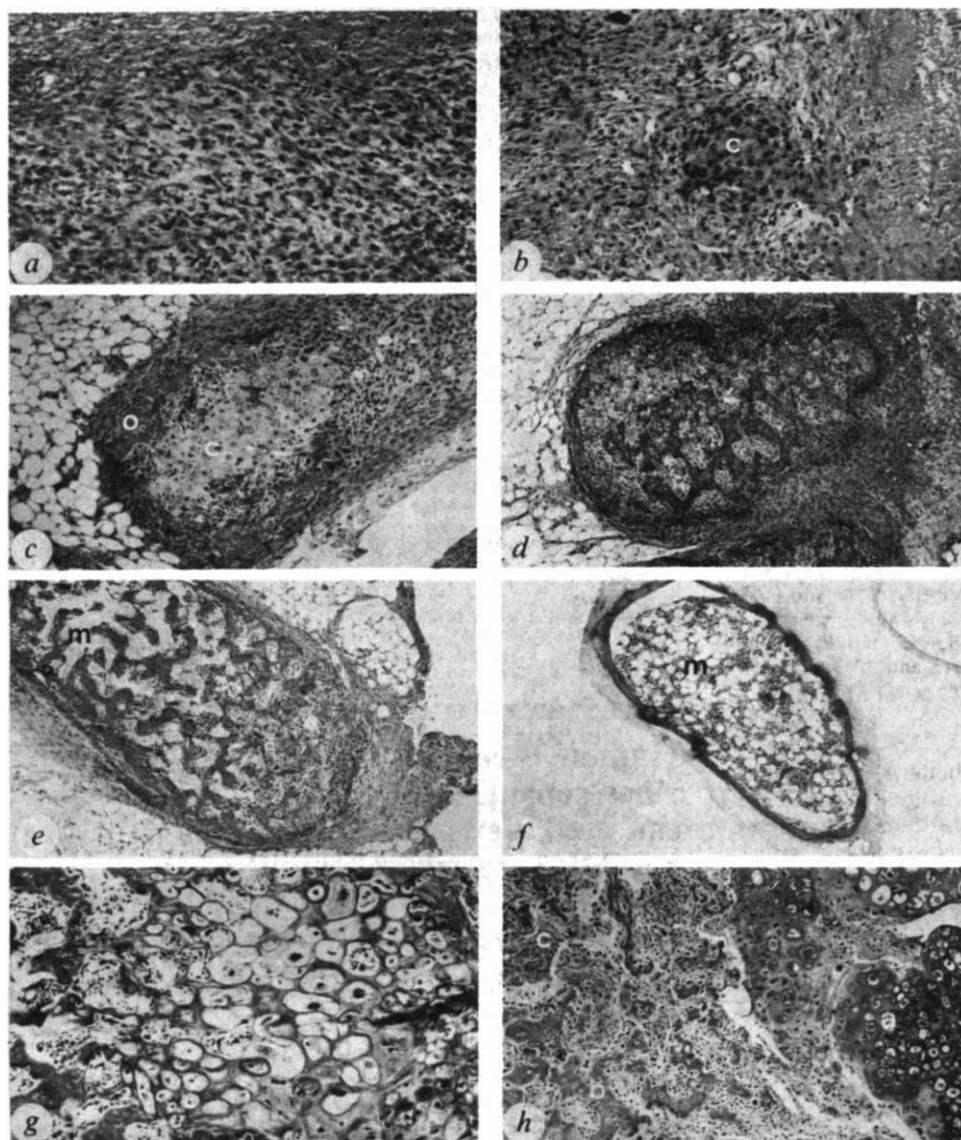


Fig. 1 Types of tumours obtained by subcutaneous injection of 3/A/1 D-1 cells. *a-f*: Ossicle formation. Detectable tumours appear 9-14 days after subcutaneous injection of cells. Final ossicle (as shown in *f*) is found 2-3 weeks after injection. *a-e* illustrate earlier aspects of tumours dissected shortly after their appearance. *a*, Primitive mesoblast (12-day old tumour); *b*, precartilaginous nodule (10-day old tumour); *c*, ossification (o) at the periphery of cartilage (*c*) (10-day old tumour); *d*, trabeculation of the bone (13-day old tumour); *e*, formation of medullary cavity (14-day old tumour); *f*, mature ossicle with marrow (transversal section, 60-day old tumour); *g, h*, malignant proliferation; *g*, abnormal cartilage (45-day old tumour); *h*, osteochondrosarcoma. *a*, cartilage; *b*, trabecular bone; *e*, mesenchyme (90-day old tumour). Cell line 3/A/1 D-1 was isolated from embryonal carcinoma cell line PCC3/A/1 after *in vitro* differentiation^{1,7}. 2×10^6 PCC3/A/1 cells were plated on tissue culture Petri dishes (Falcon, diameter 10 cm) and kept at confluency. 25 days later, various types of mesodermal derivatives were apparent. The cells were dissociated with 0.05% trypsin and replated at different densities. One week later, the culture medium was replaced for three days by a medium enriched in KCl (1 M final), a treatment found to kill selectively embryonal carcinoma cells. After several weeks, surviving differentiated cells stopped growing. Two months later some clones began to multiply. One of these was isolated and re-cloned. It was called 3/A/1 D-1. The cells were cultivated in Dulbecco's modified Eagle medium

(DMEM) with 15% fetal calf serum (Gibco). Cells were maintained at high density (not below 10^4 cm^{-2}) and replated every 2 days after mild treatment with trypsin (0.05%). To obtain benign bone formation (F), 10^6 to 5×10^6 cells were injected subcutaneously into mice (in a total volume of 0.5 ml of DMEM). Tumours appeared not later than 2 weeks after injection, in 50% of injected mice. No delayed appearance of bone was observed in the negative mice. To obtain malignant growth (*g, h*), lesions have to be provoked by scratching at the time of cell injection. In this case, 100% of the mice present an irregular ossicle after 2 weeks. In the majority of cases, this ossicle continued to grow (*g, h*) and was found to be transplantable.

Table 1 Origin of osteosarcomas and fibrosarcomas obtained in F_1 (129 $\times$ C57BL) as shown by reinjection into parental mice

	No. of mice developing tumours			
	No. of mice injected			C57BL H-2 ^k /H-2 ^k
	F_1 H-2 ^{bc} /H-2 ^k	129/Sv H-2 ^{bc} /H-2 ^{bc}		
Osteosarcoma				
1903	2/2	2/2		0/2
1860	2/2	2/2		0/2
1969	3/3	3/3		0/3
2055	2/2	2/2		0/0
Fibrosarcoma				
1738	2/2	2/2		0/2

The tumours were dissected and dissociated by mincing with scissors in DMEM + 1% fetal calf serum. They were reinjected subcutaneously or subcutaneously and intraperitoneally using a needle of 0.8 mm diameter. The injected mice were males approximately 8 weeks old.

days after the beginning of the culture showed the presence of both host cells (39 chromosomes, 1 metacentric and 78 chromosomes, 2 metacentrics) and 3/A/1 D-1 cells (75 chromosomes, no metacentric) (Fig. 3). The latter cells behave like established cells and continue to proliferate when femoral marrow control cultures have stopped growing. A cell line (3/A/1 D-1 M) has been re-isolated from these cultures. After subcutaneous injection of such cells into 129/Sv mice, a typical ossicle colonized by marrow cells is formed. Osteosarcomas, however, have never developed from these ossicles. Similar behaviour has been observed with a 10-month old ossicle of a 129/Sv mouse. Thus, even long after the formation of the ossicle, cells derived from 3/A/1 D-1 that conserve certain of their properties can be isolated among stromal cells of the bone.

The origin of the bone cells found after injection of 3/A/1 D-1 cells into a mouse cannot be studied directly in the ossicles formed. However, it can be reasonably assumed that the osteochondrosarcomas that are frequently formed when lesions are provoked at the site of injection have the same origin as the bone cells of ossicles. Thus, we have investigated the origin of osteochondrosarcomas obtained in (129/Sv $\times$ C57BL) F_1 mice. If derived from F_1 mice, these tumours should be rejected by

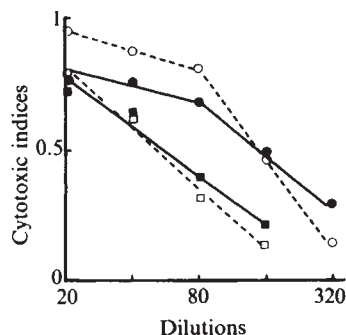


Fig. 2 Origin of ossicle marrow cells found in F_1 mice: cytotoxic effect of anti-H-2^b and anti-H-2^k sera. Cytotoxicity experiments were carried out as described in Artzt *et al.*¹⁰. Cell viability was estimated by trypan blue exclusion. Marrow cells from the ossicle were obtained by flushing the bone cavity with DMEM+5% immunoglobulin precipitated treated serum (IPT, Gibco). The cells were centrifuged, washed and red blood cells were lysed with a solution of ammonium chloride (0.87% in H₂O). After one additional centrifugation, cells were finally resuspended in Hank's+4% IPT at 10^6 cells ml⁻¹. The anti-H-2^b serum (B10D2×A) F_1 anti-B10A (2R) recognizes only private specificity 2. The anti-H-2^k serum (B10A×A) anti-B10K recognizes only private specificity 32. These two sera are a gift of Dr D. Schreffler. ●, Anti-32 serum and ■, anti-2 serum on ossicle marrow cells; ○, anti-32 serum and □, anti-2 serum on lymph node cells.

129/Sv and C57BL mice. However two tumours obtained in F_1 could be successfully transplanted in 129/Sv (but not in C57BL mice (Table 1). In several transplantations (5 to 15 transfers), these tumours retained their osteochondrosarcoma nature. Cells of one of these tumour lines have been established in culture (3/A/1 D-1 T^o). The cells are not contact-inhibited; they have an aneuploid karyotype different from that of 3/A/1 D-1 (Fig. 3). Electron microscopic analysis reveals the presence of A-type virus particles (like in 3/A/1 D-1). When injected into mice, these cells always produce osteochondrosarcomas, even after conversion into the ascitic form.

A similar analysis was done with some of the fibrosarcomas which develop when 3/A/1 D-1 cells are injected without scratching. Like the osteosarcomas, these tumours are derived from injected cells (Table 1).

In conclusion, when injected into compatible mice, 3/A/1 D-1 cells induce the formation of a structure very similar to normal bone and/or osteochondrosarcomas. All the available evidence supports the hypothesis that the bone cells are formed directly from 3/A/1 D-1 cells. Although not completely excluded, the alternative hypothesis, that is, that 3/A/1 D-1 cells induce host cells to differentiate into bone, seems unlikely.

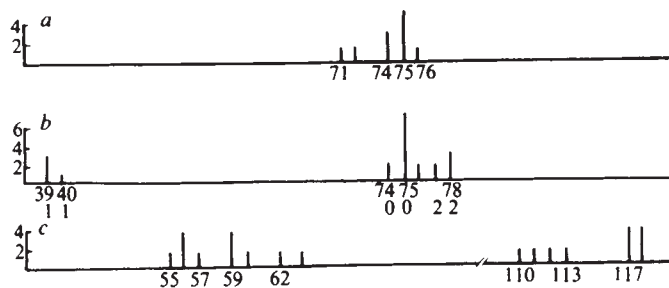


Fig. 3 Karyotype analysis of 3/A/1 D-1 (a), of stromal cells from ossicles obtained in (129/Sv×Rb 163) F_1 after 18 days in culture (b), and of 3/A/1 D-1 T^o cells (osteochondrosarcoma cells) (c). Mitotic figures were obtained as described in Rothfels and Siminovich¹¹. Rb 163 refers to strain Rb(9-19) 163 Harwell, carrying a robertsonian fusion of chromosomes 9 and 19. The number of such metacentric chromosomes is given under the number of chromosomes (lower part).

The host, however, contributes to the marrow cells which populate the ossicle. In certain conditions, malignant cells can be selected *in vivo* from such tumours. These cells may (osteochondrosarcoma) or may not (fibrosarcoma) express chondrocyte and bone potentialities. They probably derive from pre-existing variants of the cloned cells and are selected in unknown conditions. All these cell lines provide valuable model systems for the study of normal and abnormal behaviour of osteogenic cells.

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Human T-cell cultures from virus-sensitized donors can mediate virus-specific and HLA-restricted cell lysis

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Human cells infected with viruses such as herpes simplex virus (HSV) or Epstein-Barr virus (EBV) become susceptible to the lytic action of a distinct population of lymphocytes termed natural killer (NK) cells present in the blood of normal (sero-negative) and sero-positive individuals^{1,2}. Although NK cells probably represent a subset within the T-cell lineage^{3,4}, they are apparently not restricted in their lytic activity by the major histocompatibility complex (MHC) determinants as are most cytotoxic T lymphocytes (CTLs; ref. 5), but can potentially lyse a variety of unrelated virus-infected⁶ or uninfected cultured cells^{4,7}. In studies with murine models, it has been demonstrated that HSV^{8,9} or murine cytomegalovirus (MCMV) specific^{10,11} CTLs show the phenomenon of H-2 restriction initially described by Doherty and Zinkernagel with the LCM virus system¹², which is that the CTLs and targets must share H-2K or H-2D products. However, studies in man have not demonstrated the existence of HSV- or human cytomegalovirus (HCMV)-specific CTLs showing HLA (human analogue of mouse H-2) restriction in their lytic activity towards virally infected targets. We demonstrate here that T cells from the peripheral blood of patients recently cured of HSV or HCMV infections, when triggered *in vitro* with specific antigen and grown as long-term cultures in the presence of T-cell growth factor (TCGF), can mediate virus-specific and HLA-restricted lysis of virus-infected targets. We believe this to provide the first evidence that HLA-restricted and virus-specific T-cell mediated cytotoxicity can be demonstrated in human-HSV and HCMV systems. These HSV- or HCMV-specific CTLs could be important in limiting the *in vivo* spread of the respective viruses and/or in the pathogenesis of the disease caused by them.

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Table 1 Lytic activity of long-term cultures of T cells derived from individuals sensitized to HSV, HCMV or EBV towards HSV- or HCMV-infected HLA-matched or unmatched targets

Donors (HLA)	Effector T cells Seropositive for:†	Target cell donor (HLA)*	Per cent lysis of targets infected with					
			HSV-1		HCMV		Uninfected	
			50:1	20:1	50:1	20:1	50:1	20:1
Ar (A2, 32/B5, 15)	HSV-1	Autologous	42	29	16	1	2	0
		Ru (A2, 3/B15, 40)	37	24	10	0	3	0
		Sr (A1, 2/B8, 15)	40	30	12	1	2	2
		Se (A1, 9/B8, 12)	12	5	10	1	2	0
		Me (A3, 11/B7, 35)	9	5	10	2	2	0
Ru (A2, 3/B15, 40)	HSV-1	Autologous	56	25	6	3	2	0
		Ar (A2, 32/B5, 15)	39	22	9	2	0	1
		Pk (A2, w32/B15, 17)	45	27	10	1	2	0
		Se (A1, 9/B8, 12)	15	3	4	2	3	1
		Ss (A1, 29/B8, 12)	18	4	13	1	6	0
Se (A1, 9/B8, 12)	HSV-1	Autologous	39	21	4	0	3	0
		Sr (A1, 2/B8, 15)	36	26	6	1	2	0
		Ss (A1, 29/B8, 12)	32	20	9	1	2	0
		Ar (A2, 32/B5, 15)	15	3	19	2	3	0
		Ru (A2, 3/B15, 40)	9	2	10	1	2	0
Ki (A2, w24/B5, 7)	HCMV	Ar (A2, 32/B5, 45)	8	0	42	26	3	0
		Tr (A2, 3/B7, 40)	5	1	36	19	2	0
		Se (A1, 9/B8, 12)	6	0	10	2	2	0
		Ss (A1, 29/B8, 15)	12	0	9	3	1	0
Nn (A2, w24/B15, w44)	HCMV	Autologous	5	0	49	29	4	0
		Ar (A2, 32/B5, 15)	9	1	35	21	3	0
		Se (A1, 9/B8, 12)	8	0	11	2	0	0
		Ss (A1, 29/B8, 12)	16	3	8	4	0	0
Tr (A2, 3/B7, 40)	HCMV	Autologous	9	0	51	28	2	0
		Ru (A2, 3/B15, 40)	6	0	43	23	5	0
		Me (A3, 11/B7, 35)	8	1	46	29	3	0
		Se (A1, 9/B8, 12)	4	1	12	3	2	0
Me (A3, 11/B7, 35)	EBV	Autologous	8	2	9	1	0	0
		Tr (A2, 3/B7, 35)	9	1	12	2	2	0
Nk (A1, 2/B15, 38)	EBV	Autologous	6	1	8	1	1	0

Data are presented for eight blood donors; three of them (Ar, Ru, Se) had very recent (15 days–2 months) histories of herpes labialis; three (Ki, Nn, Tr) had recovered from recent (1–2 months) CMV pneumonitis and two (Me, Nk) were hospitalized for infectious mononucleosis. PBLs from heparinized blood of each donor were isolated on Ficoll–Hypaque (Lymphoprep; Nyco) gradients¹³. Washed PBL suspensions were prepared in growth medium (GM) containing RPMI 1640 (Flow), 20% heat-inactivated human serum (sero-negative for HSV, CMV and EBV), 25 mM HEPES buffer, 200 mM glutamine, 5×10^{-5} M 2-mercaptoethanol and antibiotics Aliquots (5 ml) of 10×10^6 fresh or cryopreserved PBLs sensitized to each particular virus were mixed with UV-ray inactivated samples (0, 1 μ l) of specific virus (10^6 plaque-forming units (PFU) of HSV-1 strain Thea, 10^6 PFU of MCMV strain AD 169 or 100 μ l of EBV obtained as stock virus from Dr Roggendorf, University of Munich). After 5 days at 37 °C in a humidified atmosphere with 5% CO₂, T cells were separated from stimulated PBLs as previously described^{14,15}. Briefly, washed suspension (10^7 cells ml⁻¹) was added to equal volumes of 2% washed SRBC and normal human serum pre-absorbed with SRBC. After centrifugation at 50g for 5 min, the mixture was layered on Ficoll–Hypaque, incubated at 4 °C for 30 min and then centrifuged for 30 min at 200g. Rosetting cells, that is, the T-cell enriched population, were sedimented with SRBC which were subsequently lysed by hypotonic shock and the remaining cells suspended in GM. These stimulated T cells were maintained in long-term cultures by seeding 10^5 cells in individual wells of 24-well Costra tissue culture dishes with GM plus 20% TCGF (1.5 ml well⁻¹). TCGF was prepared as described elsewhere^{16,17}. Briefly, PBL (5×10^6 ml⁻¹) from normal donors were cultured with PHA (1μ g ml⁻¹) in a total volume of 5 ml GM. After 48 h at 37 °C, the cell-free supernatant was filtered (0.22 μ m Millipore filter) and stored (–20 °C) until required. T cells maintained in TCGF had increased in density after 5–7 days and were split at 1×10^4 cells ml⁻¹ in fresh GM–TCGF. On days 15–45, the cells were washed and used as effectors in cytotoxicity assays. As targets, secondary human skin monolayer cultures from HLA-typed individuals were prepared in 25-cm flasks and inoculated with 5 PFU per cell of HSV or MCMV. After virus adsorption (37 °C for 1 h), the monolayers were washed and fed with GM containing 100 μ Ci Na₂⁵¹CrO₄. Mock-infected cultures were incubated with ⁵¹Cr as usual. After 4 h (HSV-infected) or 8 h (HCMV-infected) monolayers were disaggregated by 0.25% trypsin, washed and suspended in GM. Aliquots (100 μ l, 10^4 cells) of test targets were mixed in round-bottomed plastic tubes with 100 μ l (2.5×10^5 cells) of appropriate effectors. After centrifugation (50g for 10 min) the tubes were incubated for 4 h at 37 °C. The tubes were recentrifuged (400g for 10 min) in the cold and, from each tube, 100 μ l of supernatant was collected and counted in a γ counter. The results are expressed as % specific ⁵¹Cr release according to the formula: $100 \times (E - S)/(M - S)$, where E = release in test, S = spontaneous release in medium alone, M = maximum radioactivity releasable in c.p.m. in 1% Triton. During the 4-h assay period the leakage of ⁵¹Cr from HSV- or CMV-infected or uninfected targets in the presence of medium alone ranged from 8–12% of detergent-releasable counts. Since standard errors of triplicates seldom exceeded 3% they are omitted from the data.

* HLA-A/B antigens shared with effectors are underlined.

† Patients sensitized to HSV were negative for HCMV and vice versa.

Heparinized blood was obtained from selected donors sensitized to HSV-1 (three subjects), HCMV (three subjects) and EBV (two subjects). Peripheral blood mononuclear leukocytes (PBL) were purified over Ficoll–Hypaque density gradients by centrifugation¹³. Freshly isolated or cryopreserved PBL samples from sensitized donors were stimulated with specific antigen for 5 days *in vitro*. The activated T cells were separated by rosetting with sheep red blood cells (SRBC) as described previously^{14,15}. T cells collected from each individual were maintained in long-term proliferating cultures using TCGF obtained as 48-h supernatant from phytohaemagglutinin (PHA; Wellcome)-stimulated normal PBL^{16,17}. After 20–45 days of *in vitro* culture, the proliferating T cells of each donor were tested for lytic activity against selected panels of HSV or HCMV infected targets in a 4-h ⁵¹Cr release assay (all attempts to detect the

presence of immunoglobulin-producing cells in these cultures failed). In initial experiments, target cells were prepared by stimulating normal PBL from individual donors for 4 days with PHA (T blasts). The T blasts were then infected with virus and simultaneously labelled with ⁵¹Cr. However, when the effectors were titrated against such targets, the level of spontaneous ⁵¹Cr release was very high (up to 25%). Because of this drawback (although it had no significant influence on the interpretation of the results), human skin fibroblast cultures were established from HLA typed individuals by a standard procedure¹⁸, infected with HSV-1 or HCMV, labelled with ⁵¹Cr and then used as targets.

Table 1 shows that long-term T-cell cultures originating from three HSV sensitized individuals (Ar, Ru, Se) exerted reproducibly strong lytic activity towards HSV-infected but not

HCMV-infected autologous or HLA-A/B related fibroblast targets when assayed at an effector-to-target cell ratio of 50:1. Conversely, cultured T cells from three donors (Ki, Nn, Tr) sensitized to HCMV exerted a high level of lytic activity towards HCMV- but not HSV-infected autologous or HLA-A/B-matched targets. Freshly isolated PBL from sensitized donors or those stimulated *in vitro* with specific antigen for 5 days showed lytic activity towards virus-infected targets but this was not HLA restricted (data not shown). Apparently, for the generation of virus-specific and HLA-restricted secondary CTLs, the growth of specific antigen-triggered CTL precursors in nonspecific TCGF was mandatory. Controls, in identical conditions, proliferating long-term T-cell cultures from two donors sensitized to EBV showed no preferential HLA-restricted lysis of HSV- or HCMV-infected targets; these T cells, however, could lyse EBV-infected autologous or HLA-matched B blasts to very high levels (unpublished data). Generally, T cells from normal donors (seronegative for HSV and HCMV) were difficult to maintain in continuous cultures. However, T cells from two selected sero-negative donors could be established as long-term cultures but these, although highly efficient in mediating nonspecific lysis of targets, showed no evidence of virus specificity and HLA restriction (data not shown).

It is obvious (Table 1) that both HSV- and HCMV-sensitized T-cell cultures could mediate low but significant levels of cytotoxicity against homologous virus-infected and HLA-A/B-different (unmatched) targets. However, the amounts of ^{51}Cr released in these instances are no greater than the values when the same effectors were titrated against heterologous virus-infected targets. In separate experiments (data not shown), blocking attempts with cold HSV-infected or HCMV-infected HLA-matched fibroblasts gave a reduction in cytotoxicity against specific targets, which paralleled that ascribed to virus-specific CTLs; the amount of ^{51}Cr released due to NK cell activity being unchanged.

The above observations imply that even though the bulk of lytic activity exerted by long-term cultured T cells from HSV- or HCMV-sensitized donors was virus-specific and HLA-restricted, they also sometimes display significant nonspecific NK-cell-like activity. From the data it can be visualized that lowering the E:T cell ratio to 20:1 tended to eliminate the nonspecific lysis of targets and thereby gave generally low but exclusively virus-specific and HLA-restricted lytic values. We are now attempting to clone the long-term cultures of T cells containing potentially virus-specific and HLA-restricted CTLs in an attempt to study in detail the role of HLA-A and/or -B specificities in the recognition phenomenon.

In human-viral systems, HLA restriction of CTL-mediated cytotoxicity has been previously reported to occur only against influenza^{19,20} and measles^{21,22} virus infected cells. The issues of specificity and HLA restriction in the EBV system is still unresolved^{23,24}. The cytotoxicity data presented here have revealed HLA restriction of lysis mediated by secondary HSV- or HCMV-specific long-term T-cell cultures. However, because of the small number of sensitized donors used for establishing virus-specific T-cell cultures, one might question whether the HLA-restricted lysis mediated by them is a general phenomenon or, as in the case of the influenza virus system²⁵, donors carrying certain HLA gene product(s) lack the ability to generate virus-specific CTLs. This aspect is under investigation. Some data have been obtained (K.K.S., manuscript submitted), which suggest that for the *in vitro* generation of secondary HSV or HCMV-specific CTLs, human Ia-like HLA/D antigens have an essential role. The precise reason(s) for the failure to demonstrate the presence of virus-specific and HLA-restricted CTLs in *in vivo* situations during primary or secondary infections remains unclear; it is probably not due to the lack of development of CTL precursors during primary virus infections. In this connection, it has been shown in the mouse-HSV model²⁶ that the generation of CTLs is prevented by suppressor cells which can be eliminated by treatment of animals with cyclophosphamide.

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IgM-autoantibodies against isologous erythrocytes also react with isologous IgG(Fc)

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The mechanisms by which the adaptive immune system of animals discriminate between foreign and self components are not known. It is generally believed that antibodies are produced only in response to foreign agents. However, against this axiom there is mounting evidence that normal animals produce autoantibodies against a variety of self components^{1–6}. In two murine model systems, a high proportion of IgM-producing cells secrete autoantibodies. In one model, the autoantibodies are specific for isologous mouse IgG(Fc)⁵ and in the other, the autoantibodies are specific for antigens revealed on the surface of mouse erythrocytes (RBC) by proteolytic enzymes⁶. The function of these two autoimmune responses is a mystery. Here we show that the two responses are related. The lysis of mouse RBC, modified with bromelain (brom), by IgM autoantibodies was completely inhibited by isologous IgG(Fc), and antibodies against mouse IgG(Fc) formed precipitin bands with solubilized membranes from mouse RBC. We conclude that mouse RBC membranes and IgG(Fc) have at least one antigenic determinant in common and that this determinant is the target for autoimmune responses in normal mice.

Plaque-forming cell (PFC) assays of mouse spleen cells secreting antibodies were performed by the liquid monolayer system of Cunningham and Szenberg⁷. The methods for modifying RBC with brom and for estimating the haemolytic activity of sera for mouse brom RBC have been described elsewhere⁸. The low levels of circulating IgM directed against mouse brom RBC in normal mice were increased by injections of lipopolysaccharide (LPS), an established polyclonal B cell activator⁹.

The possibility of an antigenic relationship between mouse IgG and mouse brom RBC was first noted in an assay for the haemolysis of mouse brom RBC. Antisera against mouse brom RBC were collected from mice 4 days after an intraperitoneal injection of 40 µg of LPS. Incubation of the antisera with mouse IgG before adding the target RBC reduced its haemolytic titre from 512 to less than 2 (Table 1). The inhibition of haemolysis

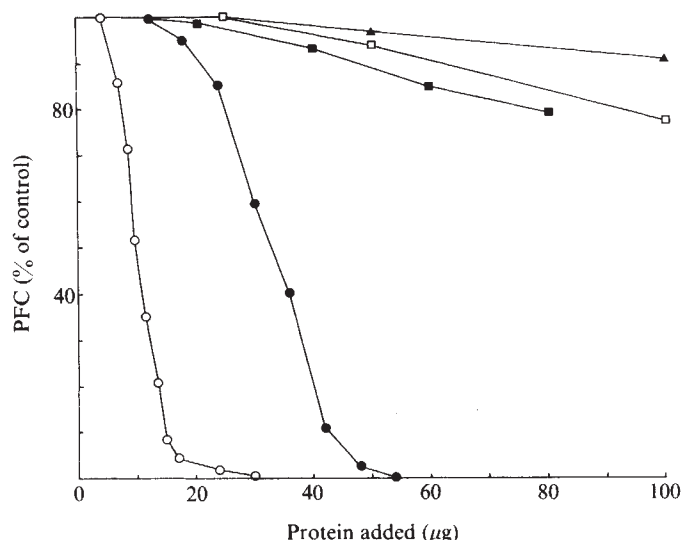


Fig. 1 The inhibition of PFC by isotologous IgG(Fc). The effect of IgG on PFC directed against three target RBC, mouse brom RBC, sheep RBC and rat RBC, was measured. To lyse mouse brom RBC, spleens were collected from normal mice while for the latter targets spleens were collected from mice 4 days after an intraperitoneal injection with either 0.2 ml 6% sheep or rat RBC. The PFC assay was performed using a reaction mixture with a final volume of 200 μ l⁸. Rabbit serum was used as the complement source for all assays. PFC against mouse brom RBC: ●, + IgG; ○, + IgG(Fc); ■, + IgG(Fab); PFC against sheep RBC: □, + IgG; PFC against rat RBC: ▲, + IgG. The 100% values were 9,000 PFC/spleen against mouse brom RBC; 88,000 PFC/spleen against sheep RBC and 34,000 PFC/spleen against rat RBC.

Table 1 Inhibition by mouse IgG of the haemolysis of mouse brom RBC

Antibody source	Target RBC	Protein added		Haemolytic titre
		Identity	Amount (μ g)	
Mouse anti-rat RBC sera	Rat RBC	—	—	128
		Mouse IgG	50	128
Mouse anti-sheep RBC sera	Sheep RBC	—	—	512
		Mouse IgG	50	256
Mouse anti-mouse brom RBC sera	Mouse brom RBC	—	—	512
		Mouse IgG	50	<2
IgM fraction of mouse anti-mouse brom RBC	Mouse brom RBC	—	—	1,024
		Mouse IgG	50	<2
		Rabbit IgG	100	256
		Guinea pig IgG	100	512
		Human IgG	100	1,024
		Mouse IgG ₁	50	<2
		Mouse IgG _{2A}	50	<2
		Mouse IgG _{2B}	50	<2
		Mouse IgG(Fc)	20	<2
		Mouse IgG(Fab)	100	256

The antisera specific for mouse brom RBC, sheep RBC and rat RBC were collected from groups of five mice, 4 days after mice in each group had been injected intraperitoneally with 40 μ g LPS or 0.2 ml 6% sheep RBC or 0.2 ml 6% rat RBC. The IgM fraction was prepared from 10 ml of mouse anti-mouse brom RBC sera by $(\text{NH}_4)_2\text{SO}_4$ precipitation, followed by chromatography on sepharose 6B. The IgG component of the fraction was then further reduced by passage through a protein A-sepharose CL-4B column¹⁴. The mouse IgG subclasses and fractions of IgG from mice, guinea pigs, rabbits and humans were each prepared from individual pools of sera (10 ml) using protein A-sepharose CL-4B (ref. 14). The Fab and Fc fragments were prepared by papain digestion of serum IgG (ref. 15). Undigested IgG was removed by chromatography on sephadex G100 and the Fab and Fc fragments separated using protein A-sepharose CL-4B. The assays were performed by incubating 50 μ l of the required antibody source with 50 μ l of PBS containing the appropriate IgG for 30 min at 37 °C. After the addition of 5 μ l of 10% RBC as indicated and 10 μ l of rabbit serum (source of complement), the incubation was continued for a further 30 min at 37 °C. The haemolytic titre was recorded as the final dilution in which complete lysis of the RBC occurred.

was not due to an anticomplement effect because the haemolytic activity of mouse antisera directed against either rat or sheep RBC was not inhibited by preincubation with mouse IgG (Table 1). The failure of the IgG in the antiserum to inhibit the haemolytic activity against mouse brom RBC is puzzling. The concentration of IgG relative to that of specific antibody may be critical.

The inhibition, by mouse IgG, of haemolysis of mouse brom RBC was further examined using an IgM fraction prepared from the sera with specificity for mouse brom RBC. The IgG content of the fraction was reduced by affinity chromatography. Table 1 shows that the haemolytic activity against mouse brom RBC was inhibited by isotologous IgG of various subclasses and by IgG(Fc) but not by IgG(Fab) or by heterologous IgG. These results are compatible with the reactivity of mouse IgM antibodies specific for isotologous IgG^{5,10}.

Mouse IgG was also used to inhibit antibodies against mouse brom RBC secreted by cells in a PFC assay (Fig. 1). The effector cells were prepared from the spleens of normal mice. The inhibition was not due to a cytotoxic or anticomplement effect because PFC assays using sheep or rat RBC as targets for spleen cells from mice immunized with either RBC type were relatively insensitive to the addition of mouse IgG. Again it was shown that IgG(Fc), rather than IgG(Fab), inhibited plaque formation.

The results presented in Fig. 2 provide direct evidence of at least one antigenic determinant being common to both mouse

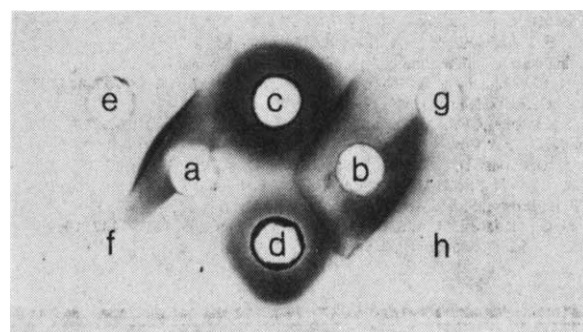


Fig. 2 Immunological cross-reactivity between mouse IgG(Fc) and mouse RBC membrane proteins. The wells contained a, rabbit anti-mouse IgG(Fab); b, rabbit anti-mouse IgG(Fc); c and d, triton soluble extracts of membranes from normal and brom RBC respectively; e and g, mouse IgG(Fab); f and h, mouse IgG(Fc). Dr P. L. Ey provided the specific sera. The method of triton-solubilization of RBC membranes has been described elsewhere¹¹. The immunodiffusion was performed in 1% (wt/v) agarose which also contained 1% (v/v) Triton X 100 and 1% (wt/v) NaCl. The gels were stained using Coomassie Blue¹⁶.

RBC membranes and IgG(Fc). Triton-soluble extracts of membranes prepared from either normal or brom-modified RBC¹¹ contained a component that reacted with rabbit antiserum specific for mouse IgG(Fc). Significantly, there was no line of precipitation between an antiserum specific for mouse IgG(Fab) and the two membrane preparations. Thus it seems very unlikely that the precipitin band between RBC membranes and anti-IgG(Fc) was due to the non-specific association of IgG with the RBC.

The biological significance of the autoimmune responses against determinant(s) common to IgG(Fc) and mouse RBC membranes is not shown by these results. One question that arises is whether both IgG molecules and RBC elicit auto-antibodies. It is possible, for example, that the primary stimulus for autoantibody production is the IgG molecule and that the autoantibodies cross-react with structures in the membranes of RBC. Thus, there may be no physiological role for the autoimmune response against RBC. If the autoimmunity is primarily against IgG(Fc), its biological role could be important in terms of functioning of immune responses. Dresser^{4,5} has proposed that IgM antibodies specific for IgG antibodies could play a role in

reacting with IgG-antigen complexes and serve to amplify the initiation of the complement cascade. However, this explanation does not account for the extremely high proportion of Ig-secreting cells from the peritoneal cavity of normal mice that are specific for mouse brom RBC. Following a short-term culture, the number of cells producing antibody against mouse brom RBC can represent up to 85% of the Ig-secreting cells present^{6,12}. It is possible that B cells in the peritoneal cavity undergo some hormonal and/or antigenic influence before being distributed throughout the body. For example, antigens present in the peritoneal cavity to which early B cells may respond could generate diversity of B cells by some, at present, unknown mechanism. Dresser⁵ has already suggested that the high level of IgM anti-IgG(Fc) secreting cells in the spleen may be involved in generating diversity. Similarly Möller and her colleagues¹³ have proposed recently that IgM antibodies against IgG(Fc) may be involved in stimulating the immune system non-specifically to generate and maintain an adequate repertoire of B cells. Experimental support for any of these suggestions would change our concept of the development and functioning of adaptive immune responses.

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A binding site on mast cells and basophils for the anti-allergic drug cromolyn

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Calcium permeability of basophil and mast cell membranes is stimulated on allergen binding to its specific membrane-bound IgE¹⁻³. This entry of Ca²⁺ ions into the cell triggers the degranulation and secretion process⁴. Disodium cromoglycate (cromolyn DSCG), the disodium salt of 1,3-bis(-2-carboxychromon-5-ylloxy)-2-hydroxypropane, inhibits the degranulation and release of anaphylactic mediators, and has found wide application in the treatment of allergic bronchial asthma⁵⁻⁷. Accumulated evidence indicates that this inhibition takes place by blocking the calcium uptake^{8,9}. To localize its site of action, the drug has been covalently conjugated to fluorescent polyacrylamide and polyglutaraldehyde beads (0.7 and 0.2 µm in diameter, respectively)¹⁰. We show here that these drug-bead conjugates (DBC) do prevent the drug penetrating into the cell without reducing its ability to inhibit histamine release (Table 1). Furthermore, we show a specific Ca²⁺-dependent binding of the DBC to the membranes of rat peritoneal mast cells (RPMC) and basophils.

Table 1 Inhibition of histamine release from rat peritoneal mast cells by DSCG and DBC

DSCG	Beads	DBC	% Release
—	—	—	3*
—	—	—	35
200 µM	10 ⁷ -10 ⁸ ml ⁻¹	—	6
—	—	10 ⁷ -10 ⁸ ml ⁻¹ †	34
—	—	—	8

Inhibition of histamine release after preincubation of sensitized mast cells¹⁴ with DSCG or DBC before antigen challenge. 2×10^5 RPMC were preincubated for 5 min in Tyrode solution at 37 °C with DSCG or DBC. Then antigen (ovalbumin 40 µg ml⁻¹) was added and the cells incubated for an additional 10 min. Histamine was assayed by the method of Shore¹⁵. Results are the mean of two experiments in which each is a duplicate.

* Spontaneous release.

† Drug/DBC $\approx 10^6$ - 10^{10} molecules per bead.

Figure 1 shows the visualization and localization of DSCG binding sites on mast cells using image intensified fluorescence microscopy. The representative fluorescence histograms obtained by fluorescence-activated cell sorter (FACS) analysis of cells stained with DBC are shown in Fig. 2. When rat basophilic leukaemia cells are incubated with DBC, areas of bright fluorescence are clearly observed over the whole cell surface (Fig. 1), and 97% of the cells are stained (Fig. 2, Table 2).

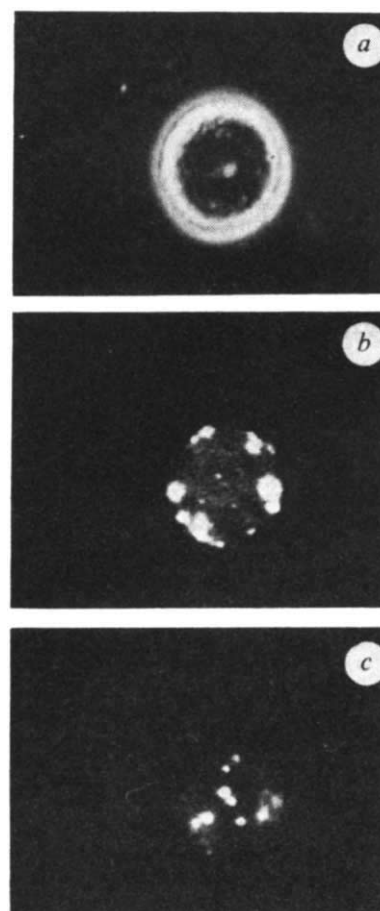


Fig. 1 Fluorescence and phase micrographs of the labelled cells were taken from a TV screen that projects an intensified image of the cells¹⁶. Rat basophil leukaemia cells (RBL-2H3) were incubated with 50 µg ml⁻¹ of DBC in Tyrode solution (NaCl 137.0 mM, KCl 2.7 mM, NaH₂PO₄ 0.4 mM, HEPES 10 mM, glucose 5 mM, CaCl₂ 1.8 mM, MgCl₂ 1.0 mM, BSA 2%) at 25 °C for 30 min. *a*, The phase mode; *b* and *c* are both in the fluorescence mode, but at different focal depths.

The possibility of DSCG acting in the extracellular fluid was excluded by the following experiment: when DSCG and Ca^{2+} ions were added to sensitized RPMC, and the cells were sedimented and resuspended in a fresh medium and exposed to antigen, inhibition of histamine release was still maintained.

Specificity of DBC binding to the cells was examined using two approaches (Table 2). One group of controls was concerned with excluding nonspecific binding of beads free of the drug. Nonspecific binding due to the extra electrostatic charges of the drug's carboxylates was tested using beads to which glutamate had been covalently conjugated. Trypsinization of mast cells (resulting in removal of membrane proteins) significantly reduced the binding of the conjugated beads. Another group of controls was concerned with the target cells' specificity of binding of DBC. BW5147 AKR/J thymoma cells and the P-815 BALB/c mastocytoma cell line, which do not secrete histamine, were not stained with DBC. Competitive inhibition of binding

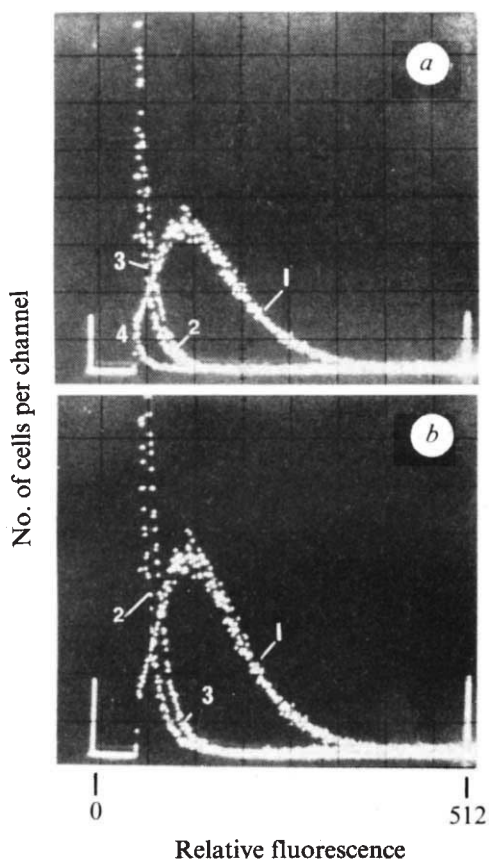


Fig. 2 Fluorescence histograms representative of rat basophilic leukaemia secreting cells (RBL-2H3) labelled with drug-tetramethyl rhodamine isothiocyanate polyglutaraldehyde bead conjugates were obtained by a fluorescence-activated cell sorter (FACS-II, Becton-Dickinson)¹³. The 400-mW, 514.5-nm line from an argon ion laser (Model 164, Spectra-Physics) intersects the stream just below the nozzle so that the cells are illuminated while they are in the fluid jet. The 580-nm large-pass filter (Dietric Optics) and the 590-nm long-pass filter (No. 2-73 Corning Glass Works) improved the red colour separation. Only living cells in the size range of 14 μm were analysed, that is, FACS was size-gated to exclude small cells (as lymphocytes) and smaller-appearing dead cells. The cells were treated as for fluorescence microscopy. *a*, Four histograms are shown: 1, specific binding of DBC; 2, cells with DBC in a Ca^{2+} -free medium; 3, cells in the presence of drug-free beads; 4, auto-fluorescence of the cells. *b*, Competitive inhibition exerted by the addition of free DSCG (see Table 2) causes a shift towards low fluorescence which resembles that due to the nonspecific staining (2), compared with the specific binding curve (1).

Table 2 DBC staining monitored by the FACS

Cells	Beads	Treatment	% Cells stained
RPMC	—	Regular	5
RPMC	Free beads	Regular	8
RPMC	DBC	Regular	71
RPMC	DBC (1)	Competition by: Preincubation with DSCG (5×10^{-3} M)	33
RPMC	DBC (2)	Co-incubation with DSCG (10^{-2} M)	19
RPMC	DBC	Ca^{2+} -free medium	6
RPMC	DBC	Preincubation with trypsin	30
RPMC	Beads conjugated to glutamate	Regular	20
RBL-2H3	—	Regular	7
RBL-2H3	Free beads	Regular	20
RBL-2H3	DBC	Regular	97
RBL-2H3	DBC	Preincubation with DSCG	32
RBL-2H3	DBC	Ca^{2+} -free medium	40
Lymphocytes	—	Regular	14
Lymphocytes	Free beads	Regular	18
Lymphocytes	DBC	Regular	14
P-815	—	Regular	4
P-815	Free beads	Regular	4
P-815	DBC	Regular	4

Each sample contained 10^6 cells incubated with DBC (10^7 – 10^8 beads ml^{-1}) in Tyrode solution for 30 min at room temperature. The RBL-2H3 cells were incubated in Tyrode containing 2% bovine serum albumin (BSA). Trypsinization of cells was carried out with a 0.25% trypsin solution for 30 min at 37 °C. Preincubation with DSCG was carried out at room temperature. As controls for staining other cells we used the BW5147 AKR/J thymoma cells for lymphocytes and also the P-815 BALB/c mastocytoma.

was found between the conjugated drug and DSCG (Fig. 2, Table 2). DSCG prevents ^{45}Ca uptake by mast cells^{8,9}. When RPMC are incubated with DBC in a Ca^{2+} -free medium, a drastic reduction of staining is observed (Fig. 2, Table 2). The phenomena reported here all demonstrate strict calcium dependence. That combined with our observation that cromoglycate forms complexes with Ca^{2+} in solvents of low polarity^{11,12}, leads us to suggest that the cromoglycate- Ca^{2+} complex binds to a specific site on the cell membrane, possibly the calcium 'gates'. For the formation of this specific ternary complex, both the cromone nucleus of the drug and its Ca^{2+} -chelating capacity are considered to be essential.

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Asymmetric F_c receptor distribution on human PMN oriented in a chemotactic gradient

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Phagocytosis of antibody-coated particles and immune complexes by polymorphonuclear leukocytes (PMN) and macrophages is initiated by binding to F_c receptors¹⁻³. While it appears that subsequent ingestion requires receptor- F_c interactions to occur along the entire periphery of the particle⁴, the distribution of F_c receptors over the leukocyte surface is unknown. We show here that during chemotaxis the F_c receptors of human neutrophils are localized to the cell's leading edge. This restricted receptor topography coincides with cell orientation and does not require net forward movement of PMN. Thus, F_c receptors are located in precisely that region of the cell most likely to first encounter opsonized particles.

IgG-opsonized sheep erythrocytes were used as F_c receptor probes. Since we found that aldehyde fixation (paraformaldehyde, formalin, or glutaraldehyde) destroyed or drastically altered F_c receptor activity, opsonized erythrocytes were bound to living cells. Incubation conditions were selected to minimize the redistribution and internalization of F_c receptor complexes⁵.

Human PMN were allowed to settle on to coverslips which were then inverted on to a chemotaxis chamber consisting of an elevated bridge between two parallel wells⁶. One well of the chamber was filled with 10^{-7} M *N*-formyl-methionyl-leucyl phenylalanine (FMLP) and the other well with Hank's balanced salt solution. A concentration gradient of the chemoattractant was thus established across the bridge. After 10–15 min, when cell orientation had occurred, coverslips were floated off the chambers with buffer, and opsonized erythrocytes at a 5%

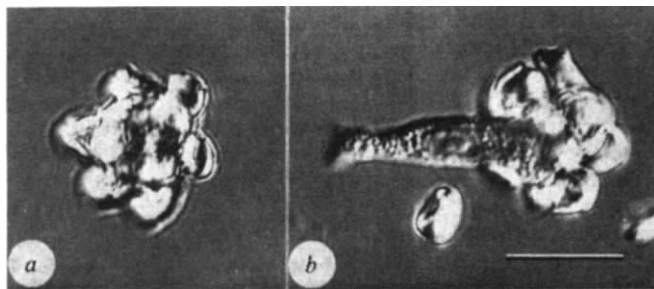


Fig. 1 Human PMN obtained by venipuncture were separated from heparinized whole blood by allowing erythrocytes to sediment at $1 \times g$ in 1.25% dextran (pyrogen-free). The leukocyte-rich plasma was drawn off, 1 ml aliquots layered on to 0.5 ml of Lymphocyte Separation Medium (Bionetics), and the gradient centrifuged in a Brinkmann-Eppendorf centrifuge for 1 min. The resulting pellet was rinsed once in Hank's balanced salt solution and contaminating erythrocytes lysed by hypotonic shock. In the final leukocyte suspension, more than 95% of the cells were PMN and the remainder were eosinophils. *a*, After settling on to glass coverslips, PMN were usually rounded or somewhat flattened. Opsonized erythrocytes bind more or less uniformly over their exposed cell surface. *b*, PMN in a gradient of FMLP for 10–15 min are oriented and motile. The nucleus is localized in the bulge of cytoplasm that marks the anterior pole. Frequently, one or more lobes of the nucleus may extend posteriorly toward the trailing uropod. In the incubation conditions used here, opsonized erythrocytes bind avidly and almost exclusively to the anterior portion of the oriented PMN. (Scale bar, 10 μ m.)

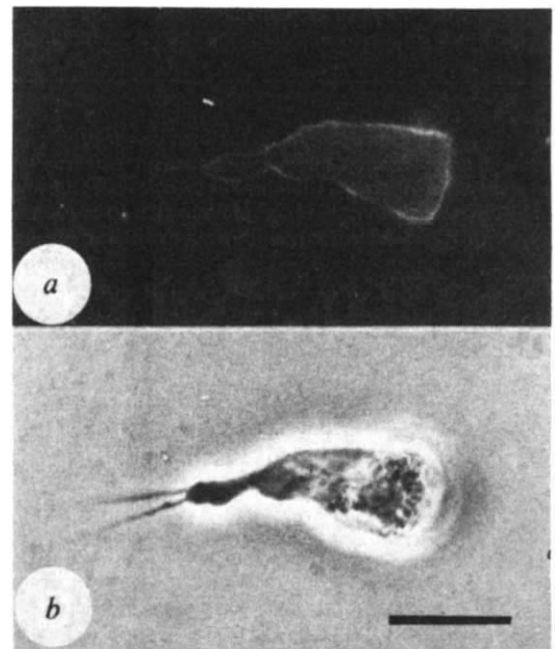


Fig. 2 PMN oriented in an FMLP gradient, fixed in 2% paraformaldehyde, and then labelled with fluorescein-Con A ($25 \mu\text{g ml}^{-1}$). *a*, Con A binding is essentially uniform except for a slight enhancement in the anterior region of the oriented cell. *b*, Phase contrast micrograph of the same PMN. Two long retraction fibres are evident posteriorly. (Scale bar, 10 μ m.)

haematocrit were placed on the coverslip for 15–60 seconds at 37 °C. Unbound erythrocytes were rinsed off and the PMN fixed with 2% glutaraldehyde. Most of the PMN (>80%) in these preparations bound one or more erythrocytes. We showed previously⁵ that little redistribution and no internalization of particle-receptor complexes occurs during this brief period of incubation at 37 °C.

The rounded or somewhat flattened non-oriented PMN (seen in the absence of FMLP) bound opsonized erythrocytes more or less uniformly about their circumference (Fig. 1*a*). Oriented PMN are elongated with the nucleus localized in a bulge near the anterior pole of the cell (Fig. 1*b*) pointing toward the well containing FMLP. A fine membrane ruffle (lamellipodium, protopod) may form the actual leading edge while a long cytoplasmic uropod trails behind the cell. More than 98% of oriented PMN that bound opsonized erythrocytes displayed them only over the anterior portion of the cell. In the few remaining cells (<2%), opsonized erythrocytes were also bound to the extreme tip of the trailing uropod. The marked asymmetry of erythrocyte binding to the leading edge of the cell is probably due to the preferential distribution of receptors, although it is possible that receptors in this region have a higher apparent affinity.

Under the conditions described above, PMN were oriented and actively motile at rates exceeding $35 \mu\text{m min}^{-1}$ along the gradient (as seen by time-lapse videotape). If calcium- and magnesium-free media containing 2 mM EDTA were used throughout the experiment, PMN became oriented with respect to the gradient but did not actually move over the substrate^{6,7}. Nevertheless, opsonized erythrocytes still bound only to the anterior pole of these oriented PMN.

F_c receptor asymmetry occurs without obligatory, parallel redistribution of other receptors. Thus, on oriented, paraformaldehyde-fixed PMN, fluorescein-concanavalin A (Con A) binds over the entire cell and shows only a slight increase in fluorescence intensity at the anterior pole (Fig. 2*a, b*). This apparent enhancement may, in fact, be caused by increased cell thickness and/or folding of plasma membrane in this region.

The mechanism for the establishment of asymmetric F_c receptor activity is not yet understood. It seems possible that coincident with cell orientation, specialized plasma membrane

domains may be set up possibly through direct or indirect cytoskeletal interactions. Microfilaments accumulate in the anterior portion of oriented PMN⁸ while centrioles along with the bulk of the microtubules are probably posterior to the nucleus⁹. This cytoskeletal architecture might directly affect the local composition of the plasma membrane^{7,9,10}. Accordingly, Fc receptors might be enriched in the anterior portion of the PMN. Alternatively, local (perhaps cytoskeleton-related) changes in the lipid composition of the membrane or in membrane surface tension¹¹ might afford regions of minimum free energy to certain classes of molecules (such as Fc receptors). In either case, Con A distribution may appear relatively unaffected by these PMN membrane domains due to the heterogeneity of the Con A binding sites.

The chief function of the PMN is to seek out and ingest invading microorganisms. This function may be facilitated by restricting the topography of Fc receptors in the membrane during chemotaxis. In particular, the movement of Fc receptors to the leading edge as the phagocyte changes orientation ensures their localization in that part of the membrane most likely to first encounter opsonized particles.

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Receptors for concanavalin A cluster at the front of polarized neutrophils

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Polymorphonuclear neutrophils (PMN) can recognize, engulf and kill microorganisms and are thus an important host defence against infection. The initial encounter between a microbe and the neutrophil takes place at the neutrophil surface. Receptors, including those for the Fc end of the immunoglobulin G (ref. 1), the C3b component of complement², formyl peptides³ and plant lectins⁴ have been identified on the surface of neutrophils. We have now examined the surface pattern and arrangement of receptors for the plant lectin concanavalin A (Con A) to determine if the surface receptor array varied with the functional state of the neutrophil. We found that receptors for Con A clustered at the front of polarized neutrophils.

Human PMN were obtained by gravity sedimentation of whole heparinized blood (10 units ml⁻¹)⁵ or by the formation of monolayers from freshly clotted blood. Cells obtained by the former approach were washed twice and resuspended in Hanks' balanced salt solution. Monolayers were prepared by incubating fresh blood on carefully cleaned glass coverslips at 37°C for 30 min. The clot that formed was washed off gently, leaving monolayers consisting of 90–95% PMN. Fluorescein- or rhodamine-labelled Con A (10 µg ml⁻¹) was added to live cells and the cells fixed in either 2% glutaraldehyde or 4% paraformaldehyde in phosphate-buffered saline. They were then examined and photographed with a Zeiss Axiomat photomicroscope under phase contrast or fluorescence illumination.

Neutrophils were considered polarized if they had a morphologically defined front (leading edge) and tail. Polarized PMN that migrated in a gradient of chemotactic substances were considered orientated. Each experiment was repeated at least three times, and in all cases 50 mM D-mannose blocked Con A binding.

PMN in suspension are spherical (Fig. 1a) with no discernible front or tail. When they were incubated with fluorescein-labelled Con A for 15 min and then examined live or fixed in suspension, a uniform surface pattern of fluorescence was observed (Fig. 1b)⁶. When PMN in suspension were fixed before the addition of Con A, the fluorescein-labelled lectin was again distributed uniformly. Thus, receptor sites for Con A were distributed uniformly over the surface of the nonpolarized PMN in suspension (Table 1).

We next examined receptor location on polarized but non-orientated PMN. PMN in suspension became polarized when incubated with the chemotactically active peptide *N*-formyl-L-methionyl-L-phenylalanine (10⁻⁶ M)⁷. Such polarized neutrophils were incubated for 15 min with fluorescein-labelled Con A and all the lectin accumulated at the tail. With prior fixation to immobilize receptor sites, there was marked accumulation of fluorescence at the leading edge in 23 ± 1.73% PMN: we call this pattern a 'headlight' (Table 1).

When cells were allowed to move across coverslips after the addition of Con A, the label moved to the tail, forming bright fluorescent buttons or 'tail lights' as described by Ryan *et al.*⁴ (Fig. 1c, d). However, when polarized cells moving over coverslips were fixed to immobilize receptor sites before the addition of Con A, the headlight pattern was seen on 69.9 ± 3.1% PMN. In other experiments, PMN were orientated towards *N*-formyl-L-methionyl-L-phenylalanine (10⁻⁶ M) in an agarose chemotaxis system⁸. Addition of fluorescein-labelled

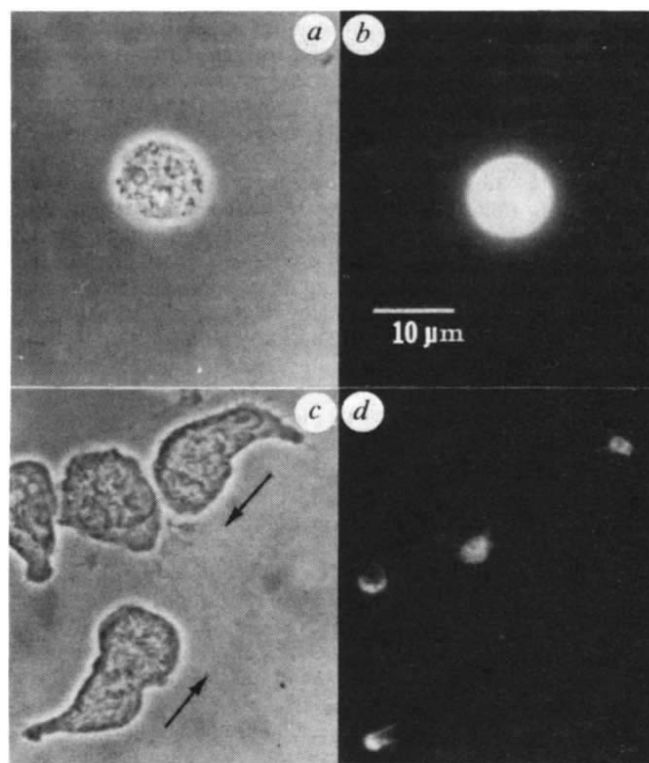


Fig. 1 Binding of fluorescein-labelled Con A to neutrophils. Each set of photographs consists of phase contrast and fluorescence micrographs of the same field. Arrows indicate direction of migration. a, b, Cells in suspension incubated with 10 µg ml⁻¹ Con A and then fixed. Binding is uniform. c, d, Live neutrophils moving on a monolayer 15 min after addition of Con A. The lectin has accumulated at the tail and thus tail lights were seen.

Table 1 Per cent of PMN with indicated distribution of fluorescein-Con A

	Head	Tail	Uniform
Nonpolarized in suspension	0	0	91.4 ± 1.2* (n = 5)
Polarized in suspension			
Fixed before Con A	23.0 ± 1.7	0	77.1 ± 1.7 (n = 3)
Fixed after Con A	0	90.8 ± 1.7	9.2 ± 1.7 (n = 5)
Polarized on monolayer			
Fixed before Con A	69.9 ± 3.1	0	30.3 ± 3.2 (n = 8)
Fixed after Con A	0	91.7 ± 2.3	8.3 ± 2.3 (n = 3)
Polarized and oriented on monolayer			
Fixed before Con A	76.9 ± 2.6	0	22.8 ± 2.5 (n = 20)
Fixed after Con A	0	91.0 ± 1.5	9.0 ± 1.5 (n = 3)

Each experiment represented counts of 100 neutrophils. *n* is the number of times the experiment was carried out on different days. Results are expressed as the mean ± s.e.m.

* Remainder showed irregular clumping of fluorescence.

Con A after fixation revealed binding sites localized at the leading edge of 76.9 ± 2.6% PMN (Fig. 2*a-d* and Table 1).

To determine whether new Con A-binding sites were available after bound sites had been swept to the tail, a monolayer of PMN labelled with fluorescein-Con A was incubated for 15 min to enable the lectin to move to the tail. After fixation, rhodamine-labelled Con A was added. Binding sites were revealed by the formation of new headlight areas of red fluorescence (Fig. 2*e*).

Incubation of PMN with colchicine (10⁻⁴ M), which interferes with microtubular function⁶, did not prevent the formation of head- or tail lights. However, incubation with cytochalasin B (5 µg ml⁻¹), a microfilament poison⁹, prevented polarization and abolished head and tail light formation. Nonpolarized PMN in suspension or on monolayers did not cap Con A receptors unless the microtubular system was first disrupted^{6,10} but, as reported here, polarized neutrophils clustered unbound Con A receptors at the leading edge and spontaneously capped bound receptors at the tail.

Oliver *et al.*¹¹ observed a uniform pattern of microfilaments redistribute to polar aggregation when associated with Con A capping in diamide-treated nonpolarized PMN. Berlin *et al.*¹² have shown that Con A-receptor complexes aggregated in the region of pseudopod formation in phagocytosing PMN. Immunofluorescent staining has shown the pseudopods to be enriched in microfilaments^{12,13}. The lamellapodium or leading edge of polarized PMN is also enriched in microfilaments¹⁴. This, together with our finding that cytochalasin abolished headlight formation, suggests that microfilaments are associated with receptor clustering at the leading edge.

Both Con A and certain piliated strains of *Escherichia coli* bind to mannose-sensitive receptor sites on the neutrophil surface^{15,16}. This implies that their binding sites may be identical. Piliated, but not non-piliated, *E. coli* are ingested by PMN in the absence of serum opsonins^{15,17}. Thus, these receptors may be important in the recognition and subsequent ingestion of microbes. We are attempting to localize complement (C3b) and immunoglobulin (Fc) receptors on polarized PMN, but we have been unable to find a fixative that maintains cellular morphology without destroying the receptor sites.

Thus, polarized PMN move with a leading edge studded with Con A receptors and when these receptors are bound, the receptor-lectin complex moves to the tail. New unbound receptors seem to be generated or uncovered at the leading edge. The array of receptors at the leading edge of the neutrophil may be important for its ability to function as an efficient phagocyte.

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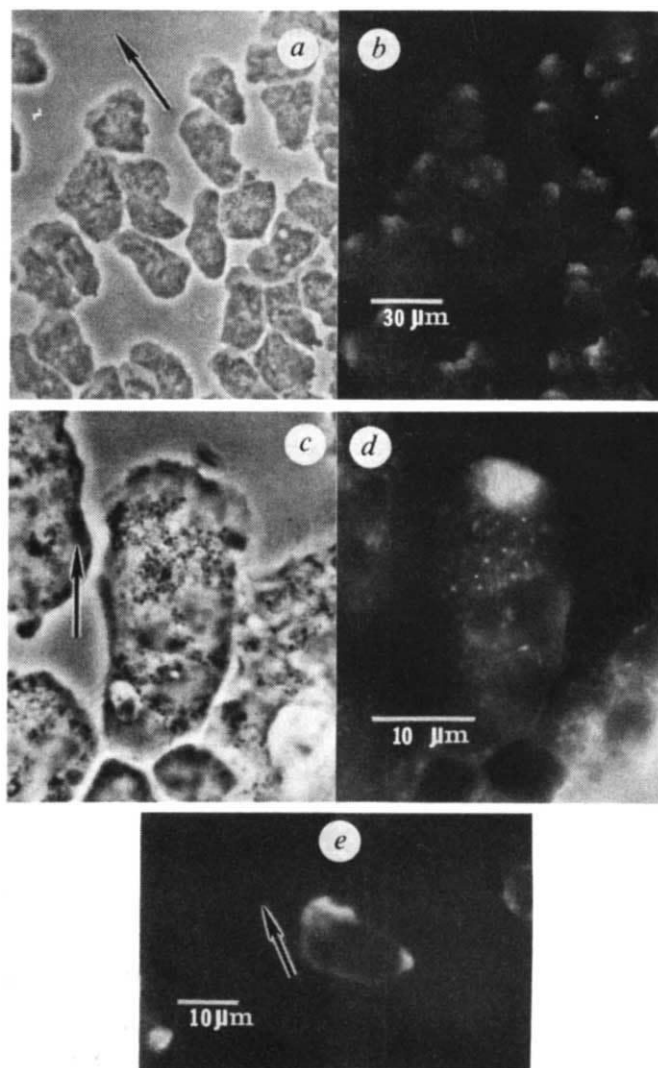


Fig. 2 Each set of photographs consists of phase contrast and fluorescence micrographs of the same field. Arrows indicate direction of migration. *a, b*, Neutrophils migrating in a gradient of the chemotactic peptide *N*-formyl-L-methionyl-L-phenylalanine under agarose for 30 min. Con A was added after fixation. A bright accumulation of Con A at the leading edge in a headlight pattern was seen on the oriented cells. *c, d*, A high power view of this phenomenon showing that Con A receptors are most dense at the leading edge. In *b* and *d* the punctate appearance of fluorescence inside the cell, not associated with the headlight, represents nonspecific granular fluorescence that is not blocked by 50 mM D-mannose. *e*, Neutrophils were incubated with Con A on a monolayer for 15 min and then fixed. Fluorescein-Con A (green in original) accumulated at the tail as in Fig. 1*d*. Rhodamine-labelled Con A was then added. Rhodamine-Con A (red in original) bound to the leading edge. Thus, the cell fluoresced in two colours, red at the leading edge and green at the tail. This indicated that new binding sites for Con A were available. These may be newly uncovered or regenerated sites.

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Topographical distribution of coated pits

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The internalization of a wide range of biologically significant macromolecules, particularly low density lipoproteins (LDL) and proteins such as α_2 -macroglobulin and epidermal growth factor, occurs primarily through characteristic, bristle-coated indentations of the cell surface known as coated pits^{1–4}. Current interest has focused on the topographical relationship between surface receptors for these ligands and the coated pits². We establish here that coated pits are themselves distributed non-randomly on the J774.2 mouse macrophage cell surface. Morphometric and statistical analyses of cell profiles in electron micrographs indicate two levels of asymmetry: in all cells, pits are clustered; after colchicine treatment they accumulate over a microvillous protuberance that develops at one pole of the cell. This topographical heterogeneity suggests a new level at which cellular responses to growth substances and hormones may be regulated.

Coated pits were defined as regions of membrane indentation approximately 0.3 μm long and underlain by a fuzzy coat which in favourable micrographs appeared as periodic spikes or bristles (Fig. 1). They frequently occupied the bases of microvilli and were characteristically separated by a clear space from the adjacent cytoplasm. To determine their distribution, a cell was first selected and photographed at $\times 8,900$. Pits with openings to the medium were then located by searching the entire perimeter of the cell at a magnification of $\times 160,000$. Their approximate positions were marked on a sketch which guided subsequent identification of pits on the low magnification micrograph of the same cell (Fig. 2a, arrows). A sonic digitizer was used to determine the length between pits and the total cell circumference from these micrographs.

Untreated J774.2 macrophages⁵ that were grown in Dulbecco's modified Eagle medium with 20% horse serum, fixed in suspension and processed for electron microscopy as before^{6–8}, maintained a rounded shape. Cell profiles were thus selected at random except that each one contained a portion of the cell

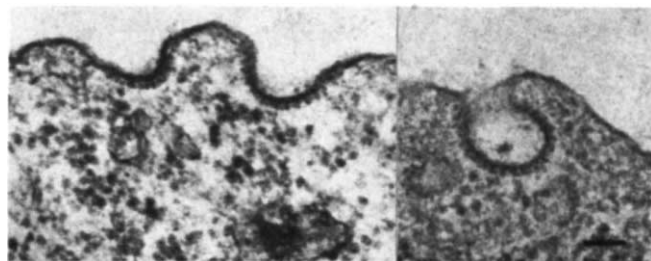


Fig. 1 The ultrastructure of coated pits. The left frame illustrates the typical thickened membrane and bristled cytoplasmic face of coated pits. The right frame demonstrates the clear zone between coated pits and the adjacent cytoplasm. Scale bar, 0.1 μm .

Table 1 Statistical analysis of coated pit distribution

Rejection of randomness hypothesis test		Untreated macrophages	Colchicine-treated protuberant macrophages
Basic quadrat*	Quadrats	NS	$P < 0.05$
χ^2 analysis	6 or 10 μm		
Basic quadrat*	Quadrat	$P < 0.01$	
χ^2 analysis	3.6 μm		
Variance/mean*		NS	$P < 0.001$
ratio analysis			
Eberhardt statistic†		$P < 0.01$	$P < 0.005$
Contiguous quadrats‡		$P < 0.005$	$P < 0.005$

* These are essentially comparisons of the observed distribution of coated pits with a theoretical Poisson distribution into quadrats. Quadrats are equal-length segments along the surface profile scored for numbers of pits. The theoretical distribution is based on the mean number of pits per quadrat. In our experiments, 37–38 pits were distributed along 600–900 μm of profile, in quadrats 3.6, 6 and 10 μm long. For the basic quadrat, χ^2 -test is used, and for the variance/mean ratio analysis, $t = (\text{variance/mean}) - 1/(2/n - 1)^{1/2}$. Both tests are insensitive to fine-grained non-randomness and are affected somewhat by the choice of quadrat size.

† In this analysis a series of random points (taken from a table of random numbers) was placed on the extended profile and a distance X_i measured from the random point to the nearest pit. The Eberhardt statistic

$$A = m \sum_{i=1}^m X_i^2 / \sum_{i=1}^m X_i^2$$

where m = number of random points used, was then computed, and its critical values taken from the table of Hines and Hines⁹. This is a sensitive test for non-randomness, particularly for clustering over small dimensions.

‡ This quadrat analysis is based on the hierarchical design of Greig-Smith¹⁰. It allows analysis at various dimensional levels, systematically 'searching' for a quadrat size that reveals non-randomness. The modification of Mead¹¹ allows analysis over a series of scales. NS, not significant.

nucleus. In these untreated cells, the average distance between pits in two separate experiments involving 37 pits on 12 cells and 45 pits on 10 cells was, respectively, 24.2 μm and 18.6 μm . Assuming the thickness of the section to be 70 nm and the cell-surface area to be roughly 1,500 μm^2 , this corresponds to a surface density of approximately 0.35 pits per μm^2 or approximately 500 coated pits per cell.

After colchicine treatment (2 μM , 30–45 min), greater than 80% of J774.2 cells developed a bulge or protuberance that was bordered by microvillous membrane and was frequently separated from the cell body by a constricted neck region^{6,8}. In these cells, analysis of randomly selected profiles yielded 14.7 μm between pits (one experiment; 37 pits on 11 cells). Analyses of profiles that displayed both the protuberance and cell body of the same cell gave 15.3 μm and 11.6 μm between pits (two experiments involving 63 pits on 12 cells and 80 pits on 11 cells, respectively). This corresponds to an average linear density of around 0.53 pits per μm^2 in colchicine-treated macrophages. As the mean pit densities on randomly chosen profiles and profiles of clearly protuberant cells are so similar, selection of protuberant profiles for subsequent studies does not introduce counting bias. The 50% increase pit density after colchicine (0.53 compared with 0.35 pits per μm^2) is unexplained but may reflect the decreased pinocytosis observed previously⁸ in colchicine-treated J774.2 macrophages.

Are coated pits distributed randomly? To answer this question the consecutive distances between pits were measured on 10–12 profiles from the untreated and colchicine-treated samples in each of two experiments. The perimeters were connected end to end by joining them at a point taken midway along the greatest inter-pit distance on each profile. This provided one continuous sample of pit distribution. A variety of statistical tests for randomness were then applied.

The distribution of pits on untreated, rounded cells was first examined statistically by dividing the total profile length into consecutive sections or quadrats of equal length, determining the number of pits in each quadrat and calculating the proportion of quadrats with different numbers of pits (0, 1, 2, 3). This was compared by χ^2 -test with a theoretical Poisson distribution based on the mean number of pits per quadrat, or by *t*-test using the method of variance/mean ratio. When the quadrat size was chosen to be 6 μm of profile, a random surface distribution of coated pits on rounded cells was indicated (that is, the statistical hypothesis that the sample is random is not rejected by these tests). When the quadrat was reduced to 3.6 μm the pit distribution appeared non-random (Table 1). This difference in statistical significance dependent on the length of quadrat suggested that the nonrandomness was based on the presence of clusters of pits. To test this, we used published methods of mathematical ecology in which nonrandomness of populations may be analysed at several scales without bias. These methods are based either on nearest-neighbour analysis, by examining the variance of distances from random points to pits along the profile⁹, or by an hierarchical quadrat analysis in which the variance at each level of complexity (scale) in relation to the next is directly examined^{10,11}. The results of these more refined tests, also summarized in Table 1, reinforce the simpler analysis indicating that coated pits are distributed non-randomly.

In contrast to the subtle clustering of pits on untreated cells, coated pits along profiles from colchicine-treated cells showed a non-random distribution by even the simplest statistical tests (Table 1). Furthermore, from inspection of the electron micrographs of protuberant cells (for example, Fig. 2a), it was suggested that coated pits were relatively more numerous in the protuberance than in the cell body. A procedure was therefore developed to combine data from different profiles so as to emphasize common topographical features. A radial grid was placed over the cell with its centre across the 'neck' region separating the protuberance from the cell body. The same grid was placed over a diagrammatic cell (Fig. 2b). Every coated pit from successful cell profiles was transferred into 1 of the 12 segments of the generalized profiles to build up a cumulative distribution pattern. The results of two such experiments are shown in Fig. 2c and d. Few coated pits are found on the 'body', whereas both 'head' and 'neck' regions are significantly enriched for coated pits. It can be seen from Table 2 that the linear density in the head is approximately five times that of the cell body. An eight-fold higher density of coated pits exists in the neck than in the cell body. This increased pit density in the protuberance and concomitant depletion of pits over the body surface is reminiscent of the capping of concanavalin A-receptor complexes on protuberant leukocytes^{6-8,12} or the segregation of surface immunoglobulin into the uropod of lymphocytes¹³.

The mechanism by which coated pits are clustered and capped is unclear. The distances between pits are too great for clustering due to ligand bridging of pits. Direct connections between pits

Table 2 Distribution and regional linear density of coated pits on profiles of J774.2 macrophages

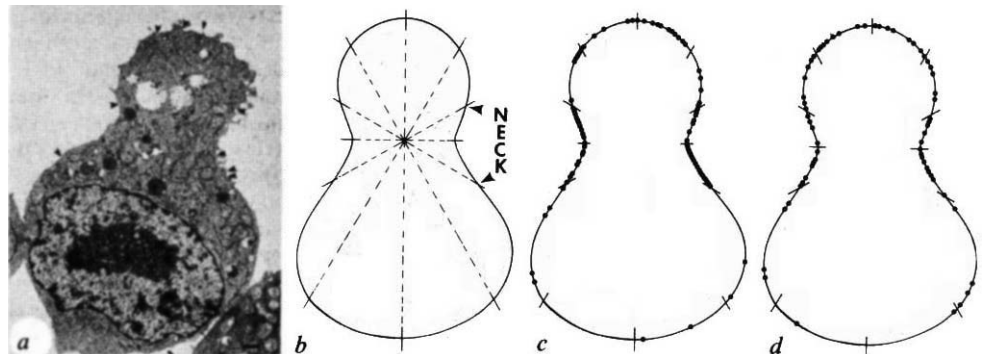
		Linear density	
Region	% Total pits	Pits per μm of profile	Relative density
Expt 1			
Control			
Whole perimeter	100	0.0413	1.00
Colchicine-treated			
Whole perimeter	100	0.0654	1.58
Head	28	0.0734	1.78
Neck	56	0.1562	3.78
Body	16	0.0187	0.45
Expt 2			
Control			
Whole perimeter		0.0595	1.00
Colchicine-treated			
Whole perimeter	100	0.0862	1.45
Head	36	0.1225	2.06
Neck	53	0.1870	3.14
Body	11	0.0238	0.40

and microfilaments that could pull them are also not necessarily involved. We have shown before that segregation of surface markers can probably occur without direct connections of the segregating species with microfilaments^{6,8,12,14}. In addition, coated pits are characteristically underlain by a clear zone that seems to lack microfilaments, although at some point microfilaments may still be involved in the internalization of coated pits¹⁵. Based on our previous work on the segregation of membrane ligand-receptor complexes^{6,8,12,14}, we propose that pits (or their molecular precursors) probably move by diffusion or by inclusion in membrane flow to overlie the neck and protuberance where they are trapped by local alterations in membrane structure, the latter perhaps being stabilized by microfilaments. However, other explanations are not excluded.

We emphasize that the existence of such topographical variation may represent a critical mechanism by which cells can modulate their responses to growth substances and hormones. Several investigators have stressed the inherently random distribution of membrane receptors of hormones that are internalized through coated pits¹⁻⁴. It follows that the clustering of pits on rounded cells and particularly their redistribution following colchicine may place coated pits at a variable distance from membrane receptors and so may alter the cellular response to ligands that bind these receptors. For example, topographical phenomena may underlie the potentiation by colchicine of DNA synthesis induced in serum-starved, quiescent fibroblasts by epidermal growth factor, fibroblast growth factor, insulin and other hormones^{16,17}.

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Fig. 2 The topography of coated pits on protuberant cells. Coated pits were first identified on electron micrographs of thin sections through protuberant cells (arrows, a). A radial grid was then placed over the profile with its centre at the midpoint of a line across the narrowest 'neck' region between cell body and protuberance and radii fanning out every 30° above and below the line. An identical grid was placed over a diagrammatic cell (b) whose 'head' and 'body' profiles were drawn in proportion to their average lengths on the individual cell profiles. This device allowed the ready transfer of pit distribution from the original cell profile to the generalized cell. The four segments referred to as 'neck' are marked: segments above these are the 'head' and segments below the 'body'. c, d Show the cumulative pit distributions from two separate experiments that analysed 12 (c) and 11 (d) profiles of colchicine-treated protuberant cells. The diagrams were built up of 63 and 80 pits, respectively. Scale bar 1 μm . Obviously, an analogous diagram for untreated symmetrical cells cannot be constructed because there is no common morphological feature by which to orientate the different profiles.



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Insulin and antibodies against insulin receptor cap on the membrane of cultured human lymphocytes

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When insulin interacts with its target cells, it binds to a specific membrane receptor and triggers a chain of events which stimulate many physiological responses^{1,2}. Although the mechanism of insulin action remains uncertain^{2,4}, electron microscopy has suggested that unoccupied insulin receptors appear in clusters of two to six molecules and that insulin becomes internalized after binding to them^{5,6}. Insulin receptors on 3T3 fibroblasts labelled with rhodamine–insulin (R-insulin) are diffusely distributed and mobile on the cell surface, with a lateral diffusion coefficient $D \sim (3-5) \times 10^{-10} \text{ cm}^2 \text{ s}^{-1}$. At 37°C, hormone–receptor complexes aggregate into immobile patches which are soon endocytosed^{7,8}. A new tool for such studies has been provided by the discovery of autoantibodies to the insulin receptor in the sera of certain patients with insulin-resistant diabetes⁹. These antibodies stimulate various physiological responses normally mediated by insulin^{10–14}. Both the bivalent antibodies (Fab')₂ and the monovalent Fab' block insulin binding, but only the bivalent antibodies are biologically active¹⁴, Fab' acting as a competitive antagonist¹⁴. Similarly, antibodies raised against purified insulin receptors can mimic insulin¹⁵, suggesting that microaggregation of receptors could be involved in the action of this hormone^{14,16}. We now report that at 37°C both R-insulin and the rhodamine-labelled antibodies against insulin receptors form a single cap on one pole of the cell. Cells which were first treated at 37°C with fluorescein–insulin (F-insulin, at partial receptor occupancy), fixed with formaldehyde and then treated with either R-insulin or rhodamine-labelled anti-receptor antibodies, showed overlapping caps of the two markers. These experiments demonstrate directly the aggregation of insulin receptors and suggest either that insulin receptors are multivalent towards the hormone or that the unoccupied receptors migrate together with the occupied ones in the formation of caps.

When IM-9 lymphocytes were incubated with R-insulin (50 ng ml⁻¹) for 90 min at 4°C, the hormone appeared diffusely distributed over the cell surface. If these cells were subsequently incubated at 37°C, the labelled hormone–receptor complexes clustered within 15 min and formed a single cap on one pole of the cell. Similar caps were observed when lymphocytes were incubated for 30 min at 37°C with R-insulin (50 ng ml⁻¹) without preincubation at 4°C (Fig. 1a, b). Insulin-induced capping occurred in approximately 60% of lymphocytes, the rest showing weak diffuse or patchy staining. Pretreatment of cells with 10⁻² M sodium azide for 30 min at 37°C inhibited capping, and R-insulin formed visible patches over the cell surface (Fig. 2a, b). Capping was not affected by preincubation for 30 min at 37°C with 10⁻⁵ M colchicine, a microtubule-disrupting agent, concanavalin A (Con A, 20 µg ml⁻¹), which inhibits capping and mobility of various other antigens on the membrane of lymphocytes, or 10⁻⁴ M cycloheximide, which inhibits protein synthesis.

When lymphocytes were labelled with 50 µg ml⁻¹ of rhodamine-labelled autoantibodies against insulin receptors, similar results were obtained. At 37°C, the antibodies induced a single cap on one pole of the cell (Fig. 1c, d). As with R-insulin, when cells were preincubated with sodium azide, patches were seen, instead of a single cap (Fig. 2e, f). Binding of anti-receptor antibody and R-insulin was specific. If the cells were incubated with 0.4 µg ml⁻¹ of native insulin with either R-insulin (Fig. 2c, d) or rhodamine-labelled anti-receptor antibodies, a weak diffuse fluorescence was observed. This reflects nonspecific interactions between the antibodies and the cell surface and is similar to the pattern observed when lymphocytes are incubated with equivalent amounts of IgG fraction of normal human antibodies (data not shown).

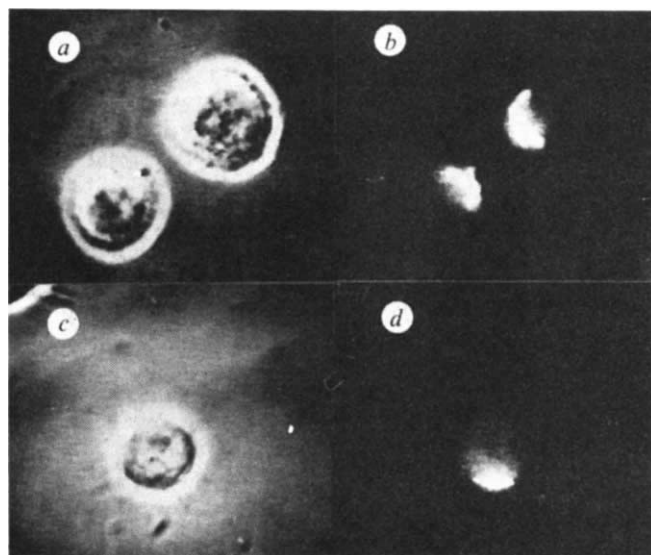


Fig. 1 The distribution of R-insulin and rhodamine-labelled autoantibodies against insulin receptors on IM-9 lymphocytes. Lymphocytes suspended in Hank's balanced salt solution (BSS) containing 1 mg ml⁻¹ bovine serum albumin (2×10^6 cells ml⁻¹) were incubated with either 50 ng ml⁻¹ R-insulin or 50 µg ml⁻¹ of rhodamine-labelled autoantibodies against the receptors isolated from insulin-resistant diabetics for 30 min at 37°C. After the incubation, the cells were washed twice with Hank's BSS, fixed with 1% formaldehyde for 10 min at 23°C, washed twice more and mounted on microscope slides for microscopic observation. The fluorescence and the phase micrographs of the IM-9 lymphocytes were taken with a Polaroid camera from a television screen that projects the intensified image of these cells. Full details about this system were previously reported^{8,28}. a, Phase; b, fluorescence micrographs of the same field of cells labelled with R-insulin. c, Phase; d, fluorescence micrographs of the same field of cells labelled with rhodamine-conjugated antibodies against insulin receptor. $\times 1,900$.

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Table 1 The distribution of R-insulin and other reagents on the cell surface

Expt	Reagents	Cell distribution	% Cells	Presence of co-caps
I	a F-insulin (5 ng ml ⁻¹)	Caps	55 ± 6	Yes
	b R-insulin (150 ng ml ⁻¹)	Caps	49 ± 8	
II	a F-insulin (5 ng ml ⁻¹)	Caps	58 ± 7	Yes
	b R-anti-insulin receptor (50 µg ml ⁻¹)	Caps	52 ± 6	
III	a F-insulin (150 ng ml ⁻¹)	Caps	62 ± 6	No
	b R-insulin (150 ng ml ⁻¹)	Diffuse background		
IV	a Insulin (150 ng ml ⁻¹)	—		
	b R-insulin (150 ng ml ⁻¹)	Diffuse background		
V	a R-insulin (150 ng ml ⁻¹)	Caps	50 ± 8	No
	b Fluorescein-lipopolysaccharide	Diffuse	100	
VI	a R-insulin (150 ng ml ⁻¹)	Caps	62 ± 11	No
	b Fluorescein-Con A	Diffuse	100	

In all the experiments the IM-9 lymphocytes were first incubated for 30 min at 37 °C with reagent *a*, washed with PBS, fixed with formaldehyde and after additional washing labelled with reagent *b* for 30 min at 23 °C. The values are the average of four different experiments.

Double-label experiments were carried out to examine whether insulin and antibody-induced receptor capping affect only the occupied receptors or produce a more general effect. Lymphocytes were incubated with F-insulin (5 ng ml⁻¹) for 30 min at 37 °C. (At this concentration 20–30% of receptors are occupied.) Cells were then washed twice in phosphate-buffered saline (PBS), fixed with 3% formaldehyde for 10 min at 23 °C and incubated for a further 30 min at 23 °C with a near-saturating concentration of R-insulin (150 ng ml⁻¹). The two markers seemed to be distributed in a single cap (Fig. 3, Table 1). When cells were labelled with saturating concentrations of either F-insulin (200 ng ml⁻¹) or native insulin (0.4 µg ml⁻¹), fixed and labelled with R-insulin, only background fluorescence was observed, in the spectral range of rhodamine. We have previously reported that hormone binding was not affected and that receptor clustering was inhibited by mild fixation with formaldehyde⁸.

A similar experiment was carried out with F-insulin and rhodamine-labelled anti-receptor antibodies. The cells were first incubated with F-insulin (5 ng ml⁻¹, 20–30% receptors occupied), washed, fixed and labelled with rhodamine anti-receptor antibodies (50 µg ml⁻¹). As before, F-insulin and rhodamine-labelled anti-receptor antibodies appeared in the same domain. Interestingly, in all these experiments, only 50–60% of cells were capped. We do not know the reason for this. It is difficult to evaluate the pattern of the diffusely labelled cells because the nonspecific fluorescence appeared in a similar pattern.

Although occupied insulin receptors may cause co-capping of unoccupied receptors, insulin-induced receptor capping does not affect the general distribution of other membrane components. IM-9 lymphocytes were labelled with R-insulin (50 ng ml⁻¹), fixed with 3% formaldehyde and incubated for 20 min at 23 °C with fluorescein-Con A (20 µg ml⁻¹). In contrast to a single cap of R-insulin observed on 62% of lymphocytes, nearly 100% of the cells showed uniform distribution of

fluorescein-ConA. Results were similar when cells were labelled with R-insulin, fixed and labelled with a fluorescein-labelled lipopolysaccharide (50 µg ml⁻¹). This molecule is embedded artificially in the lipid matrix of the membrane of various cell types¹⁷. In this experiment, R-insulin appeared in caps on 50% of the cells, whereas nearly 100% of the cells showed uniform distribution of fluorescein-labelled lipopolysaccharide.

Thus, insulin or antibodies against its receptors form patches and caps of insulin receptors on the membrane of IM-9 lymphocytes. This provides further evidence that insulin receptors can diffuse⁸ laterally in the plane of the membrane of IM-9 lymphocytes, and that insulin or antibodies against its receptors redistribute their receptors on the cell membrane. Our data also demonstrate that insulin or antibodies against insulin receptors bind to the same domain on the cell surface, as revealed by fluorescence microscopy, and that insulin receptors are operationally multivalent towards the hormone molecule. This operationally multivalent species could also be pictured as an oligomer of monovalent recognition subunits.

It is difficult to explain how a small, presumably monovalent molecule such as insulin can induce receptor clustering, although other polypeptides such as epidermal growth factor⁸,

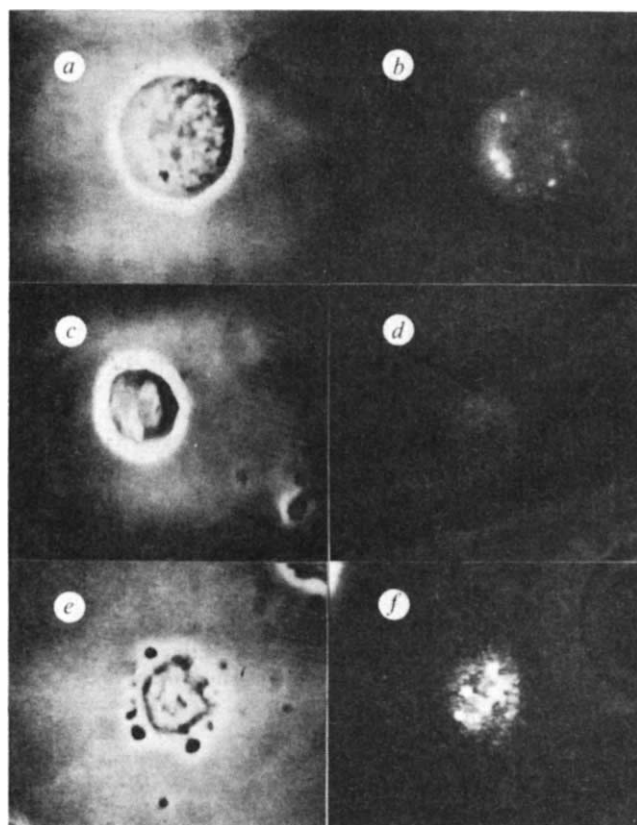


Fig. 2 Sodium azide inhibits the formation of caps of insulin or antibodies against insulin receptor on IM-9 lymphocytes. *a*, Phase; *b*, fluorescence micrographs of the same field of IM-9 lymphocytes preincubated with 10⁻² M of sodium azide for 30 min at 37 °C and then for an additional 30 min with R-insulin. The cells were washed with PBS, fixed with formaldehyde and mounted on microscope slides for microscopic observation. *c*, Phase; *d*, fluorescence micrographs of the same field of IM-9 lymphocytes which were incubated with 0.4 µg ml⁻¹ native insulin together with 50 ng ml⁻¹ R-insulin. The weak diffuse fluorescence reflects the nonspecific binding of the fluorescent hormone. *e*, Phase; *f*, fluorescence micrographs of the same field of cells preincubated with 10⁻² M of sodium azide for 30 min at 37 °C and then for additional 30 min with 50 µg ml⁻¹ of rhodamine-labelled antibodies against insulin receptor. The cells were washed, fixed and mounted for microscopic observation. ×1,900.

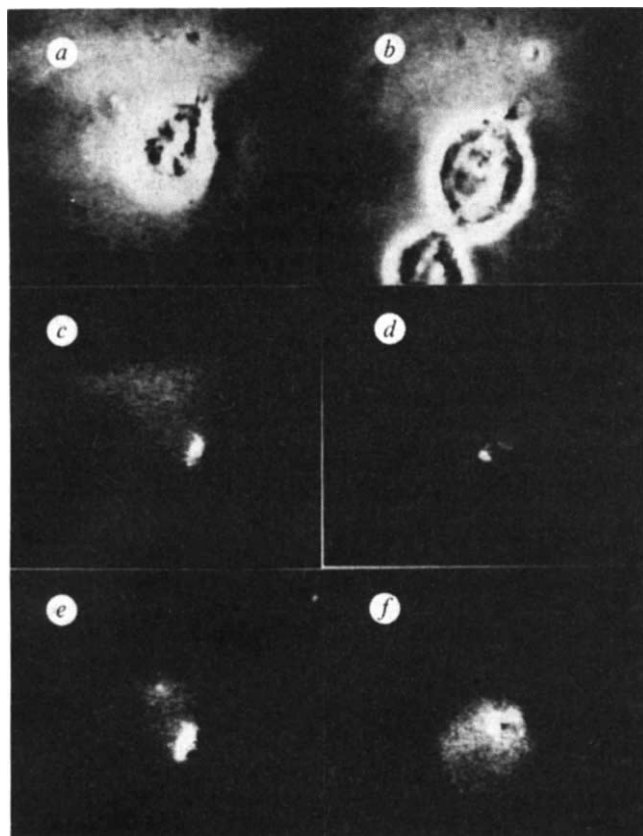


Fig. 3 Co-capping of F-insulin and R-insulin on IM-9 lymphocytes. IM-9 lymphocytes were incubated with 5 ng ml^{-1} F-insulin for 30 min at 37°C ; at this concentration 20–30% of the receptors are occupied. The cells were washed, fixed with 3% formaldehyde for 10 min at 23°C and then incubated for an additional 30 min at 23°C with 100 ng ml^{-1} of R-insulin. *a*, *b* and *c* are micrographs of one cell and *d*, *e* and *f* are micrographs of a second cell. *a* Is phase, *b* and *c* are fluorescence micrographs of F-insulin and R-insulin, respectively. *d*, Phase; *e* and *f* are fluorescence micrographs of F-insulin and R-insulin, respectively. $\times 1,900$.

nerve growth factor¹⁸ and enkephalin¹⁹ can also induce receptor clustering. For insulin, a simple mechanism would be self-aggregation on the cell surface, for insulin dimerizes and aggregates in solution at concentrations above 10^{-6} M and its local concentration on the cell membrane is about 10^{-6} M (ref. 16). On the other hand, if surface aggregation is a biologically important process, it must differ from aggregation in solution because several insulins do not aggregate in solution, but are biologically active¹⁴. A more plausible mechanism is that hormone binding induces a conformational transition in the receptor molecules that increases the binding affinity between mobile receptors. Our finding that partial occupancy of insulin receptors leads to co-capping of unoccupied receptors can be interpreted in two ways. One explanation is that the receptor molecules are multivalent towards the hormone. At low levels of receptor occupancy, one or more binding sites of each multimer is occupied, and as they cap, they bring with them the unoccupied receptors. The second possibility is that the receptor molecules are independent, but the conformational transition induced in them by the binding of insulin enhances the interactions between neighbouring receptors, leading to the migration of unoccupied receptors together with the occupied one. Both explanations, however, imply that the receptor molecule is operationally multivalent towards the hormone, which is consistent with the negative cooperativity exhibited by insulin binding to its membrane receptors in IM-9 lymphocytes²⁰. Hormone-induced microaggregation of multivalent receptors could be the mechanism of negative cooperativity. It is not known whether insulin receptors cluster in coated areas of the plasma membrane, as do α_2 -macroglobulin and epidermal

growth factor^{21,22}, and the biological significance of hormone-induced receptor capping is unclear. At physiological concentrations, insulin does not induce microscopically visible receptor caps or patches, but microaggregation of receptors is likely at such concentrations. Other data suggest that microaggregation of receptors is an important step in the chain of events leading to the bioactivity of insulin and epidermal growth factor^{14–16}. The larger microscopically visible caps are induced at concentrations of insulin in which it 'down-regulates' its membrane receptors. Indeed, we have found a good correlation between the extent of insulin-induced receptor loss (down-regulation) and the amount of IM-9 lymphocytes which cap (unpublished results). Furthermore, both processes depend on hormone concentration, time and temperature. This suggests a possible physiological role to insulin-induced receptor capping.

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Myosin from fetal hearts contains the skeletal muscle embryonic light chain

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The contractile proteins of adult skeletal and cardiac muscle tissue are very similar. Some of these proteins are identical in amino acid sequence^{1,2} or differ only slightly³. Various structural studies have shown that the heavy and light chains of myosin are also homologous in skeletal and cardiac muscles^{4–11}. In developing skeletal muscles, certain myosin subunits are present which are not found in the corresponding adult muscle^{7,10–13}. Whether fetal cardiac muscle also contains myosin subunits homologous to these early forms is not known. We now report that ventricular myosin of fetal rats has a light chain polypeptide corresponding to the skeletal muscle embryonic light chain¹¹. This result provides further evidence that a form of myosin light chain, not detectable in adult skeletal or ventricular myosin, is characteristic of the early stages of striated muscle development¹⁴.

Myosin purified from 20-day embryonic rat ventricles by Sepharose or DEAE-cellulose chromatography¹¹ contains three components of the same molecular weight as adult myosin (Fig. 1*a*), but also has a band of approximate molecular weight (MW) 26,000 (Fig. 1*b*). Two-dimensional gel analysis of adult

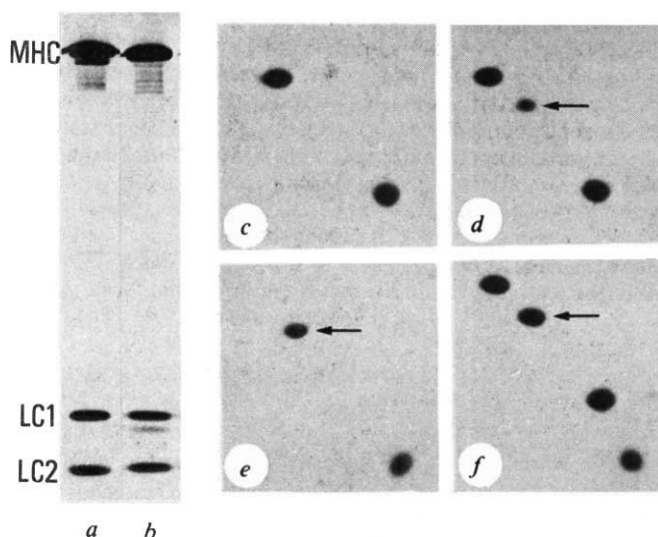


Fig. 1 Gel electrophoresis of various myosin samples. Myosins were extracted and purified from adult rat heart ventricles, 20-day fetal rat heart ventricles and fused L6 myotube cultures as previously described¹¹. The final step in the purification procedure for the preparations shown here was chromatography on Sepharose 2B in the presence of 1 mM ATP¹¹. *a*, Adult cardiac myosin and *b*, embryonic cardiac myosin were analysed on one-dimensional SDS gels. The positions of light chains (LC1 and LC2) and heavy chain (MHC) are indicated. Two-dimensional gel analysis of myosin light chains¹¹ was performed on *c*, adult cardiac myosin, *d*, embryonic cardiac myosin, *e*, L6 myosin and *f*, a mixture of L6 and embryonic cardiac myosin. Only the light chain region is shown. About 15 µg of each myosin were loaded on the gels. The arrows in *d-f* indicate the LC1_{emb} protein.

and embryonic cardiac myosin light chains (Fig. 1*c,d*) clearly revealed two and three components respectively. Myosin from the myogenic rat cell line L6 is composed of two light chains, an LC2 protein of the fast skeletal type plus the embryonic light chain LC1_{emb} described previously¹¹. Co-electrophoresis of embryonic cardiac myosin with L6 myosin showed that the third light chain component in the cardiac preparation corresponded to LC1_{emb} as determined by their co-migration (Fig. 1*e,f*). No fast-type light chains were detected in the embryonic cardiac myosin, as suggested previously¹⁵. The earlier result may have been due to contamination of the embryonic myofibrillar preparations by histones, as shown elsewhere^{16,17}.

The possibility that cardiac and L6 embryonic light chain are identical is supported by the following results. During partial proteolysis of SDS-denatured myosin using *Staphylococcus aureus* protease, LC1_{emb} is converted to a polypeptide of approximately 23,000 MW, whereas LC1 light chains of adult fast, slow and cardiac myosin are relatively resistant⁷. We have verified that this 23,000-MW species is not derived from the heavy chain at these levels of degradation by comparing the cleavage pattern of isolated L6 myosin heavy chain with that of the whole molecule (heavy and light chains; not shown). As Fig. 2 shows, cardiac LC1_{emb} is degraded to a polypeptide of the same molecular weight as that derived from the L6 embryonic light chain (Fig. 2*a,b*). The resistance of the adult cardiac LC1 to *S. aureus* protease (Fig. 2*c*) also suggests that it is not directly related to LC1_{emb}.

The ratios of the various cardiac myosin light chains were determined by densitometry of the stained bands on one-dimensional SDS gels. Adult myosin contains LC1 and LC2 in a ratio of $1.16 \pm 0.08:1$ ($n = 5$), which after correction for their respective molecular weights (22,000 and 20,000 (ref. 9)) is close to the expected 1:1 stoichiometry. Densitometry of embryonic cardiac myosin reveals that the ratio of LC1 + LC1_{emb} to LC2 is $1.19 \pm 0.08:1$ ($n = 4$). LC1_{emb} represents about 25% of the total LC1-type light chain in this myosin.

The myosin heavy chain of embryonic cardiac myosin was examined by partial proteolytic cleavage after denaturation by

SDS^{7,18}. The pattern of degradation is very similar to that of adult cardiac myosin after treatment with *S. aureus* protease (Fig. 2*b,c*). The one-dimensional analysis of patterns obtained after cleavage by chymotrypsin or papain also reveals extensive similarity (results not shown). However, two-dimensional analysis after chymotryptic degradation reveals several quantitative differences. For example, some minor polypeptides in the adult myosin pattern (Fig. 3*a*) are major species in the embryonic pattern (Fig. 3*c*); they are indicated in Fig. 3*b* by arrows in the gel containing a mixture of the two reactions. Other polypeptides are major in the adult and minor in the embryonic preparation. No detectable amounts of any adult fast or embryonic skeletal myosin heavy chain isoforms are seen in the cleavage patterns of embryonic cardiac myosin (Fig. 2*a,b*, and ref. 7). The quantitative differences seen in the embryonic and adult patterns may mean that these preparations are mixtures of two or more isoforms, the ratios of which are different. This possibility is consistent with results of electrophoresis of native intact myosins¹⁹ which reveals three rat ventricular myosin isoforms (V_1 , V_2 and V_3). V_3 is predominant in 18-day embryos (J. F. Y. Hoh, personal communication) while V_1 is the major form in the adult¹⁹. It is therefore possible that the heavy chain isoform we find in embryonic heart tissue corresponds to that of V_3 myosin. Interestingly, a V_3 isoform reappears in large amounts in hypertrophic hearts²⁰.

We have demonstrated a novel form of myosin light chain in embryonic skeletal muscle¹¹, and the results presented here reveal an identical, or at least very similar, protein in embryonic rat ventricular myosin. Because the two-dimensional gel technique is capable of high resolution, co-migration of these two proteins is suggestive of identity. The presence of a cleavage site

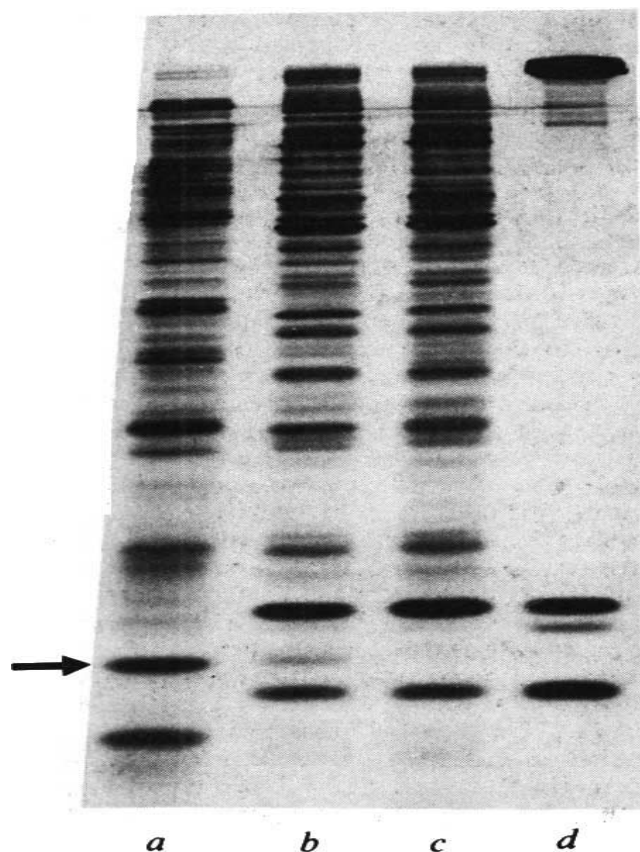


Fig. 2 Partial proteolysis of skeletal and cardiac LC1_{emb} by *S. aureus* protease. About 16 µg of myosin was treated with *S. aureus* protease in the presence of SDS as described previously⁷ and then analysed on a one-dimensional SDS gel. Results are shown for the treatment of *a*, L6 myosin, *b*, embryonic cardiac myosin and *c*, adult cardiac myosin. *d*, Untreated embryonic cardiac myosin shows the positions of various intact light chains. The LC1_{emb}-derived fragment is indicated by an arrow.

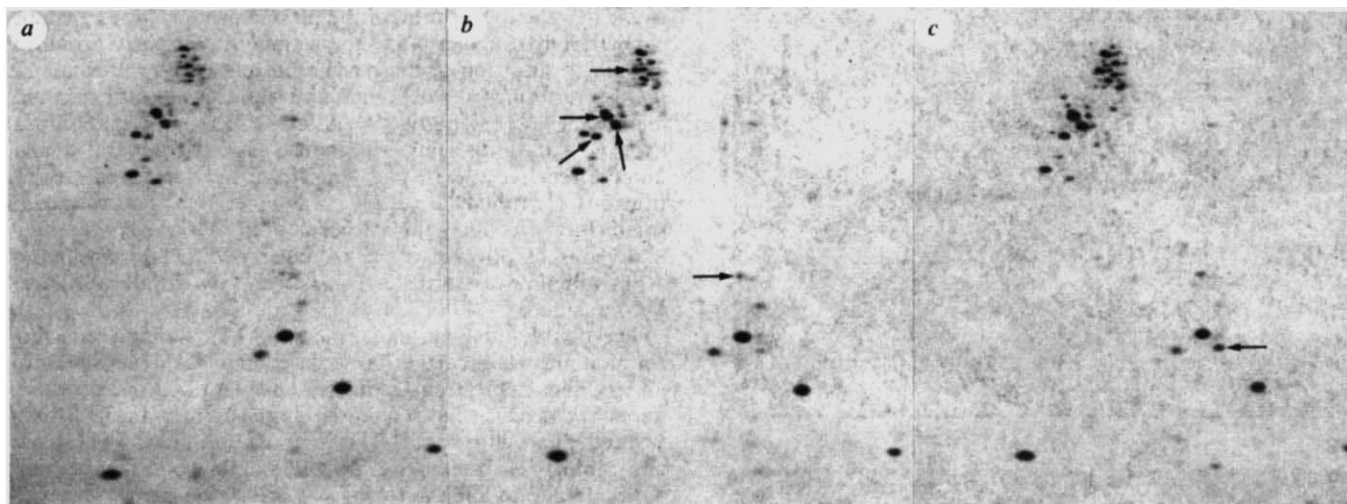


Fig. 3 Polypeptide maps of adult and embryonic cardiac myosin heavy chains. The partial chymotryptic cleavage reactions were carried out as described for two-dimensional gel analysis⁷ except that the final myosin concentration was only 1 mg ml^{-1} . About $60 \mu\text{g}$ were loaded on the gels containing *a*, adult and *c*, embryonic myosin cleavage products. Gel *b*, contained about $30 \mu\text{g}$ of each of the adult and embryonic cleavage products. The arrows in *b*, indicate polypeptides that are major in the embryonic myosin map *c*, and minor in the adult map *a*. The arrow in *c* indicates the intact LC1_{emb} protein. Two-dimensional gels were run as described¹¹ except that the isoelectric focusing gel was 21 cm long and the second dimension slab gel was 19 cm high, composed of a 4-cm stacking gel and 15-cm separation gel. The gels are presented with the basic pH range to the left.

for *S. aureus* protease in both the skeletal and cardiac proteins is also consistent with this possibility. The LC1_{emb} light chain is not found in adult ventricular myosin (shown here) nor in adult skeletal muscle¹¹. While we cannot rule out that LC1_{emb} might be a major component of some adult myosin, it seems to be present in skeletal and ventricular myosin only at the early stages of development. The relative stoichiometry of the LC1 and LC2 components in fetal cardiac myosin is the same as in adult myosin, although LC1_{emb} partially replaces the adult cardiac LC1 at the early stages.

The similarity of the ventricular heavy chain isoforms found at 20 days *in utero* with those of the adult could be interpreted in several ways. Conceivably a distinct embryonic form is never present in developing myocytes. Alternatively, cardiac tissue could be much more developed biochemically at this stage than skeletal muscle, and an embryonic form may already have been replaced by the adult forms. Even though myocytes are still proliferating in the perinatal period, these dividing cells are biochemically differentiated²¹. In contrast to the heavy chain, the light chains have not attained the adult phenotype by 20 days of gestation. If the absence of a distinct embryonic heavy chain at this time is simply due to rapid turnover of such a form, then the situation is the inverse of developing skeletal muscle in which LC1_{emb} disappears more rapidly than embryonic heavy chain^{7,14,22}.

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Coexistence of α -fetoprotein, albumin and transferrin immunoreactivity in neurones of the developing mouse brain

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α -Fetoprotein (AFP), a major fetal plasma protein, is present at high levels (mg ml^{-1}) throughout development and at extremely low levels (ng ml^{-1}) in the normal adult (see refs 1,2). Unlike that of most other species, rat and mouse AFP bind oestrogens with a high affinity³. This aroused speculation regarding its involvement in the growth and differentiation of oestrogen target tissues⁴. Extracellular sequestration of maternal oestrogens through binding to AFP is generally assumed to protect the female rodent brain from excessive oestrogenization (masculinization) from the high oestrogen levels present during the critical period for sexual differentiation⁵. There is an intracellular pool of AFP of unknown function in brain cytosol of fetal and neonatal rats⁶ and mice⁷. Its intraneuronal localization has been confirmed within the central and peripheral nervous systems of the developing rat^{8,9} and human fetus¹⁰ by immunoperoxidase cytochemistry. Other major plasma binding proteins such as albumin^{9,10} and prealbumin¹⁰ but not transferrin^{9,10} have also been localized within neurones of the developing rat and human brain. As part of a continuing ontogenetic study, I present here the first evidence, derived by single- and double-label immunofluorescence, for the intraneuronal localization and coexistence of immunoreactive AFP, albumin and transferrin within the same neurones of the late fetal and postnatal mouse brain. These observations may be relevant to the process of sexual differentiation of the brain, and the mechanism of transport of hormones and other growth-promoting substances into neurones during development.

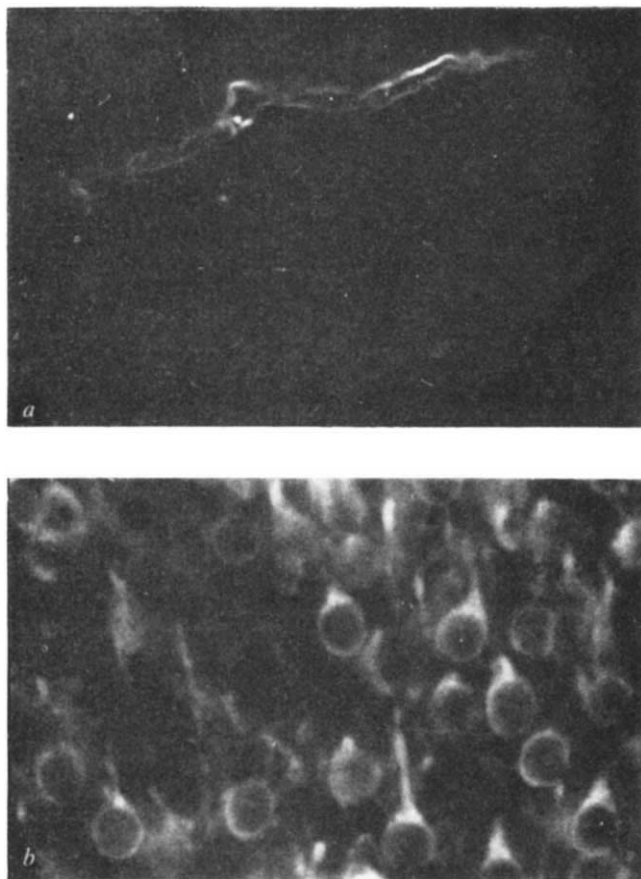


Fig. 1 *a*, Immunofluorescent staining of the newborn male mouse hypothalamus with the non-immune IgG control. Note the total absence of cellular fluorescence and the localization of the staining to a blood vessel only. This section was adjacent to the one in Fig. 2*b* $\times 100$. Detailed methods for Figs 1 and 2 are given in the text. *b*, Immunofluorescent localization of mouse AFP in immature neurones of the 18-day fetal female mouse cerebral cortex. Note the cytoplasmic immunoreactivity with extension into the primary neuronal processes. $\times 175$.

Brains from sexed 18-day fetal, newborn, 8-day post-natal and adult mice were immersed in ice-cold ethanol/acetic acid¹¹ without stirring for 24 h, dehydrated, cleared and embedded according to the method of Sainte-Marie¹², and processed for single- or double-label immunofluorescence. Demonstration of antigen localization was carried out by incubations of 3–4- μ m serial coronal sections with purified goat antibodies to mouse AFP, the γ -globulin (IgG) fraction of rabbit antiserum to mouse albumin either unlabelled or conjugated to fluorescein isothiocyanate (FITC) (Cappel), or goat antiserum to mouse transferrin (Cappel) for 48 h at 4 °C. After washing in phosphate-buffered saline (PBS) (pH 7.4), antibody binding was visualized either by the direct method (FITC-conjugated anti-mouse albumin) or indirectly by adding goat anti-rabbit or rabbit anti-goat IgG conjugated to either (FITC) (green) or tetramethyl rhodamine isothiocyanate (TRITC) (red) (Cappel) for 24 h at 4 °C. The sections were then washed in PBS (pH 7.6), mounted in Gelvatol and examined with a Zeiss microscope equipped with both fluorescein and rhodamine optics, and epi-illumination.

Controls included: (1) non-immune goat and rabbit sera; (2) absorption (neutralization) of AFP antibodies by newborn mouse serum (high AFP levels); (3) absorption with purified mouse albumin or mouse transferrin (Cappel); (4) the fluorochrome conjugates alone, and (5) newborn mouse liver, a site of AFP, albumin and transferrin synthesis, as a positive control. These showed no fluorescence except for the liver, which

confirmed the localization of the plasma proteins to the parenchymal cells. Furthermore, to distinguish the specific intracellular localization of the plasma proteins from their passive uptake, resulting from experimentally induced alterations in cell membrane permeability^{13,14}, parallel sections were always exposed to antiserum to mouse IgG (Cappel)¹³. IgG immunoreactivity was usually localized only to blood vessels (Fig. 1*a*). Neuronal fluorescence was generally absent, although small clusters of IgG-staining cells were occasionally seen only in regions of obvious tissue damage. Cells were considered AFP-, albumin- or transferrin-positive only if they were also IgG-negative.

The double-labelling experiments (AFP-albumin; transferrin-albumin) were carried out by a combined direct (albumin) and indirect (AFP; transferrin) method. This approach became necessary because the sequential application of the two conjugated fluorochromes (TRITC-rabbit anti-goat; FITC-goat anti-rabbit) resulted in the double staining of each primary antibody due to apparent conjugate cross-reactivity. The conjugated fluorochromes themselves, however, did not detect the inappropriate antibody nor did they stain the tissue in the absence of antibody. The double labelling experiments consisted of sequential applications on the same tissue section of the goat antibodies (AFP or transferrin), TRITC-rabbit anti-goat IgG and FITC-rabbit anti-mouse albumin IgG, each for the time periods described above. Double-label detection of AFP and transferrin was not possible because of their shared species of origin (goat).

Intracellular localization of all three plasma proteins was observed only in the brains of the 18-day fetal, newborn and 8-day postnatal mice. The adult brains exhibited no fluorescence. Specific fluorescence was observed in cell groups throughout the developing brains of both sexes and seemed to be localized to neurones only. Large numbers of post-mitotic neuroblasts and immature neurones exhibited bright, specific intracytoplasmic fluorescence which often extended into primary processes, initial dendritic segments and axons (Figs 1, 2). Neuronal nuclei did not fluoresce. AFP-, albumin- and transferrin-containing neurones were widely distributed in diverse regions, including the cerebral cortex (Fig. 1*b*), septum, basal ganglia, hippocampus, brain stem (Fig. 2*a*), hypothalamus (Fig. 2*b*), cerebellum and medulla. The numbers of fluorescing neurones in any given region increased with its developmental stage. This was exemplified quite strikingly in the cerebral cortex, where the numbers and topographic distribution of fluorescing neurones were closely correlated with differentiation of the cortical layers. In the hypothalamus, preoptic area and the amygdala, however, certain nuclear regions known from ³H-oestradiol autoradiography to contain oestradiol receptors were rendered anatomically distinctive by the complete or almost complete and bilaterally symmetrical absence of fluorescence. These regions include the medial preoptic area, the suprachiasmatic, arcuate, ventromedial and ventral premammillary nuclei of the hypothalamus, and the medial and cortical amygdaloid nuclei and will be described elsewhere (in preparation). Specific fluorescence was also seen in the epithelial cells of the choroid plexus, in the meninges, in blood vessels, to varying degrees in the apical portions of ependymal cells, and in the extracellular space of the developing brains. Fluorescence of the extracellular space, however, decreased significantly between birth and the 8th postnatal day and was totally absent from the adult brain.

Double-labelling experiments demonstrated that all neurones which exhibited fluorescence for either AFP or transferrin (red) also contained albumin (green). Although the lack of suitable antibodies precluded any direct comparisons of AFP and transferrin localization, the topographic distribution of albumin-containing cells in serial sections suggests that all three proteins are in the same neurone.

This is the first demonstration of AFP, albumin and transferrin immunoreactivity in the developing mouse brain and of their coexistence within the same neurones. These observations also confirm that the AFP in mouse brain cytosol is intraneuronal⁷.

Furthermore, transferrin has not previously been localized intraneuronally, its specific absence, rather, being noted^{9,10}. Because of the inadequacy of the blood-brain barrier at the ages studied, the intraneuronal coexistence of the three plasma proteins makes it seem more likely that they have been taken up by endocytosis from the cerebrospinal fluid and blood¹⁰ than synthesized locally. This hypothesis is supported by the observed immunoreactivity of blood vessels, choroid plexus epithelium and the extracellular space. To investigate the alternative possibility, however, we are now determining whether or not AFP is synthesized in developing neurones by carrying out *in situ* and solution hybridizations to the messenger RNA with a cloned chromosomal DNA probe specific for AFP.

The discrete intracytoplasmic localization of AFP, albumin and transferrin raises questions regarding their possible active and essential role in neuronal differentiation and development. As binders of a variety of hormones and other biologically active substances, these three plasma proteins may mediate or modulate their intraneuronal transport. It has been proposed, for example, that at low steroid concentrations cellular entry of oestrogen into the immature rat uterus may be a carrier-mediated event in contrast to the diffusion seen with high levels¹⁵. Moreover, the essential role of albumin in the active transport of thyroid hormones into rat hepatocytes in culture has been demonstrated¹⁶. Incorporation of albumin (but not of other proteins) into the nutrient medium of cultured chick sensory ganglia, furthermore, significantly enhanced the biological activity of nerve growth factor (NGF) by doing more than merely preventing its adsorption to glassware¹⁷.

The intraneuronal localization of the oestrophilic AFP has important implications for the process of sexual differentiation of the rodent brain. Its intracellular presence forces one to

reconsider the generally held concept that the female rodent brain is protected from exposure to maternal oestrogens by the extracellular AFP⁵. On the contrary, neuronal uptake (perhaps by endocytosis) of such oestrogen-binding proteins as AFP and albumin must bring oestradiol into the cell. Intracellular dissociation of the protein-oestradiol complex, resulting from the greater affinity of oestradiol for its receptor, could liberate the steroid for subsequent association with its cytoplasmic receptors. Such a mechanism might thus provide certain neurones of both sexes with the intracellular source of oestradiol of non-androgen origin previously postulated as necessary for the induction of both male and female patterns of neural differentiation^{18,19}.

This phenomenon, however, need not be limited to the particular proteins described here. In those species (birds, humans) whose AFP has little or no oestrogenic affinity^{1,2}, other binding proteins, including albumin, may serve the same functions by similar mechanisms. Its possible active role in the transport of thyroid hormones and NGF-like substances, among others, as well as its capacity to bind oestrogens, renders albumin also of considerable potential importance to the development of the central nervous system. Furthermore, the intraneuronal localization of albumin makes it imperative that antisera raised to bovine serum albumin-conjugated antigens be pre-absorbed with albumin before use in immunocytochemical studies of developing central nervous system tissue both *in situ* and in cultures exposed to serum-containing medium.

The significance of the intraneuronal localization of transferrin is unknown. That transferrin *per se* may mediate important developmental functions, however, is suggested by recent *in vitro* observations²⁰ that transferrin (90% iron-free) is essential for neuronal differentiation and survival in serum-free medium.

The cellular uptake of plasma proteins during development may not be unique to the brain. Preliminary studies (unpublished observations) suggest that immunoreactive AFP, albumin and transferrin are also localized within cells of the neonatal adenohypophysis. Their presence in other hormonal target tissues remains to be determined.

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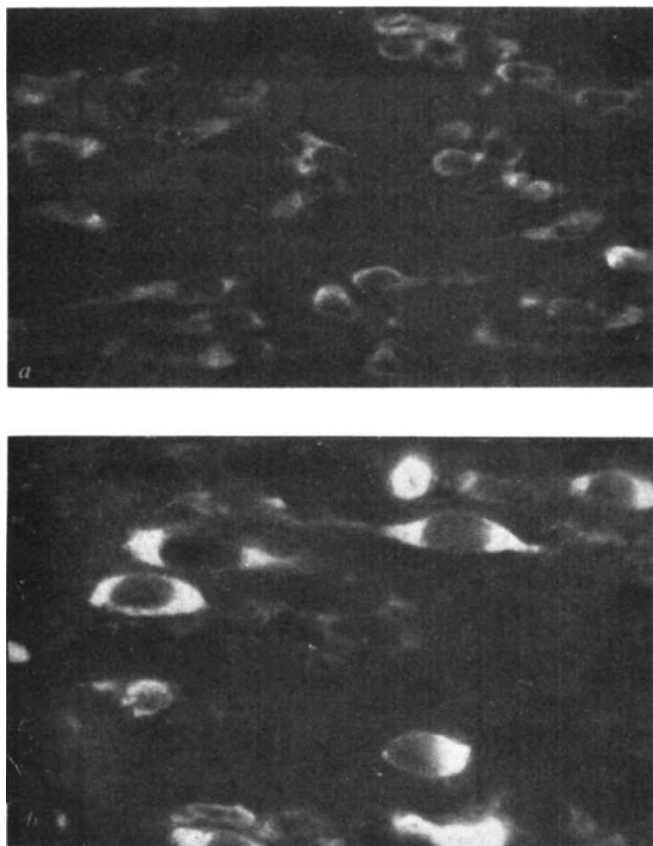


Fig. 2 *a*, Immunofluorescent localization of mouse albumin in neurones of the 8-day female mouse midbrain. $\times 100$. *b*, Immunofluorescent localization of mouse transferrin in neurones of the newborn male mouse hypothalamus. $\times 175$.

Glial cells in the enteric nervous system contain glial fibrillary acidic protein

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The complex nervous networks found throughout the mammalian gut—the enteric nervous system—are histologically, ultrastructurally, and, to some extent, functionally—similar to the central nervous system¹⁻³. The glial cells of the small enteric ganglia are generally classified as Schwann or satellite cells, since they are found in the peripheral nervous system, possess nuclei which ultrastructurally resemble those of Schwann cells and are derived from the neural crest^{4,5}. However, it has been argued that these cells resemble astrocytes of the central nervous system with respect to gross and fine structure, and their relationship with the enteric neurones and their processes^{1,6}. In immunohistochemical studies of these cells, both in frozen sections of gut wall and in tissue culture preparations of the enteric plexuses, we found evidence that the enteric glial cells are rich in glial fibrillary acidic protein (GFAP), a protein associated with the 100 Å glial intermediate filaments⁷, and hitherto believed to be specific to astrocytes of the central nervous system only^{8,9}.

Figure 1 illustrates the immunofluorescence seen when frozen sections through ganglia of the rat myenteric plexus, cerebellum and sympathetic ganglia were treated with antisera raised against human GFAP; similar results were obtained with three different antisera^{9,10}. As shown in Fig. 1b, the antisera reacted strongly with the glial cells in the myenteric ganglia, the

immunofluorescence characteristically surrounding the neuronal cell bodies, which were always unstained. The glial staining was of similar intensity to that of astrocytes in frozen sections from the central nervous system (Fig. 1b, d). No staining was found in the Schwann cells, satellite cells, neurones or axons in sections from other autonomic ganglia with any of the three antisera (Fig. 1c). However, Yen and Fields have recently found evidence that a minority of peripheral glial cells in adult rat sciatic nerve sections also contain GFAP immunoreactivity using the same antisera as we used here (personal communication).

It has been demonstrated that astrocytes of the central nervous system maintain their glial filaments when grown in tissue culture and show striking, filamentous GFAP immunofluorescence on treatment with GFAP antisera¹¹. It was therefore interesting to see whether enteric glial cells in culture also exhibited GFAP immunoreactivity. Explant cultures of the myenteric plexus were obtained from the proximal colon of 7–14-day-old rats as described elsewhere¹². We have suggested that one of the cell types in such cultures, identifiable on the basis of occurrence, morphology and interaction with other cells, may be the enteric glial cells¹². When cultures of the plexus, maintained *in vitro* for 7–10 days, were treated with the GFAP antisera, it was found that most of these putative glial cells showed bright, filamentous immunofluorescence, very similar to that seen in astrocytes in tissue culture (Fig. 2). Other flat cells, fibroblast-like in morphology, were unstained, as were the neuronal cell bodies and their processes. As with astrocytes in culture¹³, the morphology of the cells showing GFAP immunofluorescence was quite varied. Many, however, had tapering processes and in most cells part of the cell body flattened out, forming often extensive, thin membraneous expansions.

To establish conclusively that the glial cells in the enteric plexuses contained GFAP, we compared the distribution of GFAP immunofluorescence with that of neurofilament (NF)

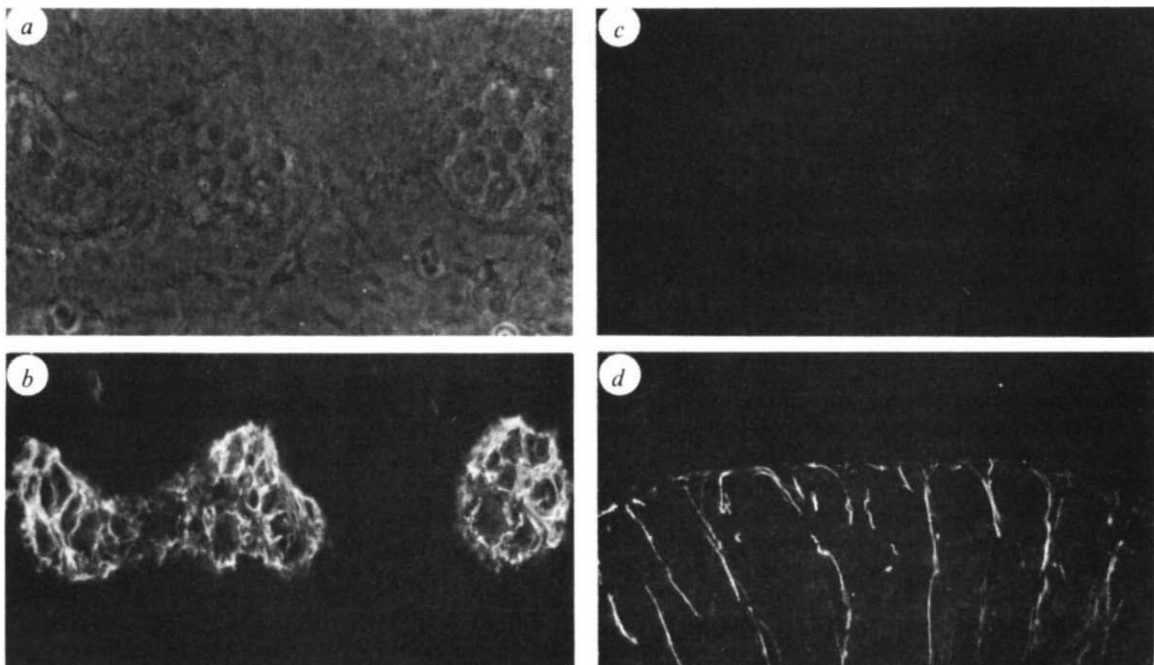


Fig. 1 GFAP immunofluorescence in frozen sections of *a* and *b*, rat colon, *c*, dorsal root ganglion, and *d*, cerebellum. 10 µm sections were left overnight at 4 °C, then incubated overnight at 4 °C with rabbit anti-human GFAP serum (1 : 1000), followed by goat anti-rabbit IgG conjugated to rhodamine (G anti-R1g-Rd) for 30 min at room temperature. They were viewed with *a*, phase contrast or (*b*–*d*), fluorescence optics, using a Zeiss Universal microscope. Only the glial cells in the myenteric plexus are labelled in *b*; the neurones in the plexus and the circular and longitudinal muscle are all unlabelled. No immunofluorescence is seen in any of the dorsal root ganglion cells *c* while the cerebellum shows typical GFAP labelling in processes of Bergmann astrocytes *d* (*a*, *b*, *c* ×300; *d* ×200). The antiserum donated by Drs D. Dahl and A. Bignami has been extensively characterized in a number of studies, which have shown that it specifically stains astrocytes both in tissue sections and cultures and does not stain other cells in nervous or non-nervous tissues^{8,9,11,13,16}. The antisera donated by Dr R. Pruss were prepared from human GFAP as described by Drs D. Dahl and A. Bignami⁹. Extensive testing of these antisera on tissue sections and cultures revealed that the staining properties of both antisera are identical to those of the Dahl and Bignami antiserum (R. Pruss, personal communication).

immunofluorescence. The NF antiserum was prepared against the 155,000 molecular weight subunit of neurofilament protein¹⁴. The distribution of NF immunofluorescence in the rat myenteric plexus and sympathetic ganglia is illustrated in Fig. 3. In ganglia of the plexus (Fig. 3a) the NF antiserum typically revealed the neuronal cell bodies. No fluorescence was present in the glial cells surrounding the neurones, so that the NF staining pattern was the converse of that seen with the GFAP antisera. In the superior cervical ganglion (Fig. 3b) the most prominent sites of NF immunoreactivity were the axons of transversely cut axon bundles. Staining was also seen in the cell bodies of most neurones although the intensity varied markedly between cells and only a few of them stained as intensely as did the axons. All these structures were completely unstained when treated with GFAP antisera. Thus, the pattern of NF immunofluorescence in both tissues is completely different from that of GFAP immunofluorescence, from which we conclude that the immunoreactivity observed in these tissues following treatment with GFAP antisera resides in glial cells and not in neurones.

GFAP is the major protein of the 100 Å filaments found in astrocytes; the GFAP-containing filaments are one of five different, but related classes of intermediate filaments that can be distinguished both by immunological and biochemical methods. The others are neurofilaments, found in central and peripheral nervous systems, desmin filaments, present in muscle cells, keratin filaments in epithelial cells and vimentin filaments, the only filament type that has a wide distribution among different types of cells¹⁵. GFAP-containing filaments have so far been found exclusively in astrocytes of the central nervous system and have often been used as a cell-type specific marker for astrocytes^{7-9,11,13}. However, our results indicate that under appropriate conditions, GFAP can be expressed by glial cells in the peripheral nervous system. The enteric glial cells, like astrocytes, express GFAP both *in situ* and in culture. It has previously been noted that although the enteric glial cells have some of the morphological characteristics of Schwann cells they also show notable structural similarities with astrocytes^{1,5,6}. Like astrocytes, the enteric glial cells carry extensive branching and irregular processes which mingle with neuronal processes in a dense synaptic neuropil, they possess glycogen granules and contain a very large number of glial filaments, arranged in a

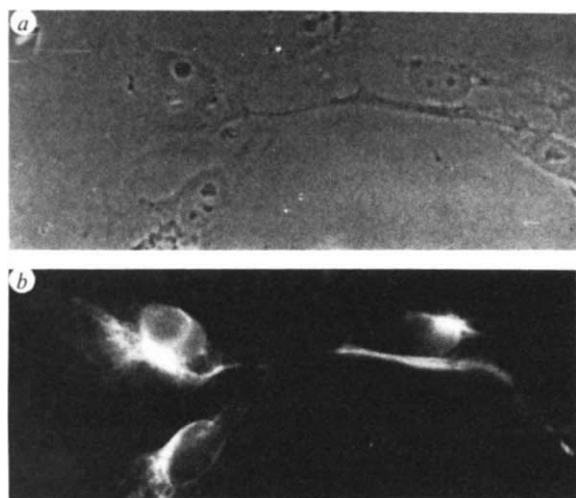


Fig. 2 GFAP immunofluorescence in explant culture from the proximal colon of 15-d-old rat. *a* Represents a phase contrast view of the same field as shown in *b* where fluorescence optics are used (see Fig. 1). Cultures were fixed with 5% acetic acid in ethanol at -20°C for 10 min, incubated with rabbit anti-human GFAP (1:100) for 30 min at room temperature, followed by G anti-RIG-Rd for 30 min at room temperature. Flat cells in the culture are stained in a filamentous pattern similar to GFAP staining of cultured astrocytes ($\times 640$).

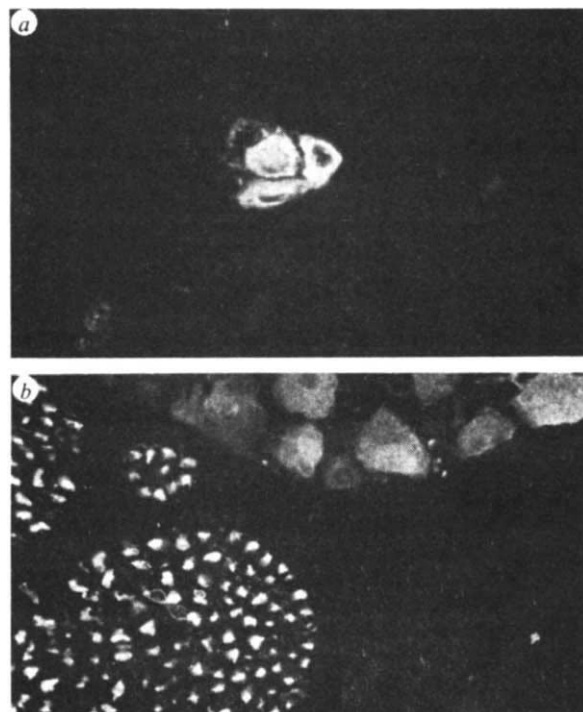


Fig. 3 Neurofilament immunofluorescence in frozen sections of *a*, rat colon and *b*, dorsal root ganglion. $10\mu\text{m}$ sections were left overnight at 4°C and then stained overnight with rabbit anti-NF (1:400), followed by G anti-RIG-Rd for 30 min at room temperature. They were then viewed with fluorescence optics as described in Fig. 1. Three neuronal cell bodies in the myenteric plexus are clearly stained with the NF antibodies (*a*). The glial cells surrounding them, in contrast to Fig. 1 *b*, are unlabelled. Neuronal labelling is seen in both cell bodies and axons in the dorsal root ganglion (*b*). In both *a* and *b* there is a clear difference in the pattern of staining between the NF antiserum and the GFAP antiserum staining seen in Fig. 1 ($\times 430$).

manner similar to that seen in astrocytes. The results described in this paper demonstrate that these enteric glial cells are different from typical Schwann cells and satellite cells not only in structural but also in some biochemical features. More work is needed to determine whether these cells should be classified as modified Schwann or satellite cells, adapted to suit a complex, integrative nervous system, or whether they are sufficiently different from other peripheral glial cells to warrant consideration as a separate class of glial cells.

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Monoclonal anti-acetylcholine receptor antibodies can cause experimental myasthenia

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Myasthenia gravis (MG) is a disease which occurs as a consequence of an autoimmune response directed against the nicotinic acetylcholine receptor (AChR) of the myoneural junction¹⁻³. Antisera raised against complex antigens such as AChR comprise a mixture of antibodies reactive with various determinants on the antigen molecule. The antibodies against any single determinant may be of several immunoglobulin classes and idiotypes. Antibodies produced by cloned lymphocyte-myeloma hybridoma cell lines have provided a way of analysing the diverse components making up a polyclonal antiserum⁴ and assessing the relative contribution made by each to the overall immune reaction *in vivo*^{5,6}. We have applied this technique to the investigation of the autoimmune response in MG. We demonstrate here that certain monoclonal anti-*Torpedo* AChR antibodies, when injected intravenously into normal rats, induce an acute myasthenic syndrome. Thus binding of a single molecular species of antibody reactive with a single antigenic determinant can result in all of the manifestations of an autoimmune disease.

Hybridoma cell lines producing anti-AChR antibody were obtained and partially characterized as described elsewhere^{7,8}. A Lewis rat (Microbiological Associates) was immunized with AChR purified from the electroplax of *Torpedo californica*. Spleen cells from this animal were fused with the mouse myeloma cell line P3-X63-Ag8 (provided by Dr C. Milstein). Proliferating hybridomas were screened for production of anti-AChR antibody by a haemagglutination assay (HA) using sheep erythrocytes coated with *Torpedo* AChR (ref. 8).

The binding properties of the hybridoma antibodies have been partially characterized by assessing changes in HA titre when AChR-coated red cells were preincubated with cholinergic ligands. Antibodies from the independently isolated, cloned, hybrid cell lines fall into four groups (1) blockable by α -bungarotoxin; (2) partially blockable by cholinergic ligands; (3) titres increased in the presence of some nicotinic ligands; (4) titres unaffected by any cholinergic ligand or by α -bungarotoxin⁸. The heavy chain class (Table 1) of each antibody was determined by radial immunodiffusion with antisera (provided by Dr Hervé Bazin)⁹. Immunoglobulin-enriched culture supernatant preparations from each hybridoma and from P3-X63-Ag8 were injected intravenously into individual young female Lewis rats (Fig. 1). Animals were examined every 12 h. At either 48 or 72 h the animals underwent *in vivo* measurement of the evoked muscle responses of the gastrocnemius muscle to repetitive sciatic nerve stimulation and *in vitro* measurement of miniature endplate potentials (m.e.p.ps) from the diaphragm¹⁰. All m.e.p.p. amplitudes were corrected to a resting potential of 85 mV. Input resistances ranged from 4 to 15 M Ω . At 48 or 72 h tissues were taken for histological study and blood samples were obtained for serum antibody titre determination by both HA and radioimmunoassay¹¹.

Two of six antibodies tested (56I and 73G) produced both the clinical and electrophysiological manifestations of myasthenia in injected animals (Fig. 1, Table 1). Injection of similar amounts of antibody (as determined by both antibody titre and immunoglobulin concentration) from the other anti-AChR producing

hybridomas or from the myeloma line alone was ineffective. Decrementing evoked muscle responses to repetitive nerve stimulation and diminished amplitude of m.e.p.p. correlated with clinical disease (Table 1). Of five animals tested with clinical signs of myasthenia, four demonstrated decrement of 10% or more. None of 10 animals injected with other antibodies or with the myeloma protein exhibited decrement. The m.e.p.p. amplitudes of 6 of 8 animals injected with antibodies 56I or 73G were lower than any of the values for the 10 animals injected with the other antibodies or myeloma protein. The means of the two groups differed significantly [0.55 ± 0.02 compared with 0.70 ± 0.02 mV (mean $\pm$ s.e.m.), $P < 0.001$]¹².

Frozen sections of muscle were obtained from several sites. Mononuclear cell infiltrates were located selectively in the regions of endplates of animals injected with antibodies 56I or 73G (Fig. 2a). In these areas numerous fibres were undergoing necrosis with frank invasion by mononuclear cells. In sections stained for cholinesterase activity, many cholinesterase-reactive regions were separated from the underlying fibres; the cholinesterase staining was sometimes diffuse and lacked detail (Fig. 2b). Frequently mononuclear cells were interposed between the muscle fibres and the cholinesterase-reactive regions (Fig. 2c). Serial sections stained for acid phosphatase demonstrated that the infiltrating mononuclear cells were macrophages. No histological abnormalities were found in animals injected with the other antibodies or the myeloma protein.

The syndrome produced here by injection of a hybridoma supernatant fraction containing monoclonal anti-AChR antibody is indistinguishable from the acute phase of rat experimental autoimmune myasthenia gravis (EAMG)¹³ or from the disease produced by injection of syngeneic immune serum¹⁴. The fact that preparations from some anti-AChR producing clones, and from the myeloma line alone, produced no effects suggests that it is not other factors present in the culture supernatants that are responsible for the observed findings. This is the first demonstration, to our knowledge, of the production of

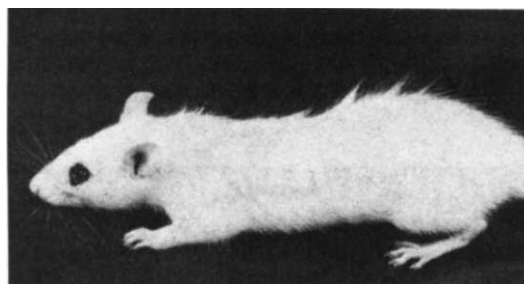


Fig. 1 Rat injected 48 h earlier with immunoglobulin-enriched fraction from hybridoma 56I. Fractions were prepared from culture supernatants of hybridomas grown in Dulbecco's modified Eagle's medium plus 20% heat-inactivated fetal bovine serum. Supernatants were concentrated 10-fold by pervaporation against powdered sucrose followed by dialysis against phosphate-buffered saline (PBS), pH 7.2. The final product was obtained by precipitation in 50% saturated ammonium sulphate, resuspension in PBS to ~5% of original volume, and dialysis against PBS. A similar fraction was prepared using supernatants from cultures of the myeloma line P3-X63-Ag8. Approximately 1 ml (40–100 mg of protein of 56I and 73G, up to 250 mg of the other antibodies) of each preparation was injected into normal rats. Agar gel electrophoresis followed by densitometric scanning indicated that all preparations consisted of 85–90% albumin and 10–15% immunoglobulin. Isoelectric focusing of the material (performed by David Mattson) revealed 8–12 immunoglobulin bands, 6 of which were those of the myeloma protein. Clinical disease had a similar time course in animals receiving either of the two myasthenia-inducing antibodies and correlated with dose of antibody injected and with serum titre at time of death. Extremity weakness was noted 18–24 h after injection; this worsened over the next 24–36 h. In more severely affected animals susceptibility to fatigue was such that, after one or two steps, the animals lurched to either side. Some showed almost no power in flexion or extension of upper extremity digits so that the front paws remained cupped. Weight loss in these animals, possibly caused by weakness of neck extensors and muscles of mastication, averaged 17 g over the 3-day period of observation. Consistently observed were dried porphyrin secretions crusting the eyelids, nares, and periorbital fur. Weakness was reversed temporarily by intraperitoneal edrophonium chloride.

a defined pathological syndrome by an immune reaction mediated by monoclonal antibody. Since the antibody is monoclonal it is presumed to bind to a single determinant on the AChR molecule, to express a single set of idiotypic determinants, and to be of a single immunoglobulin class. It must have, therefore, a single set of effector functions, for example, interaction with complement, interaction with macrophages. These characteristics are sufficient, in the cases of antibodies 56I and 73G, to induce myasthenia.

Data are not yet available to explain the failure of some of the anti-AChR antibodies to induce myasthenia. Since the antibodies were raised against AChR from *Torpedo californica* electroplax, it is most likely that the ineffective antibodies simply do not cross-react with rat junctional AChR (preliminary data seem to support this explanation). Other possible explanations may include a requirement for binding to a particular portion of the AChR molecule.

Mere binding of antibody to AChR is probably not sufficient to induce the syndrome. Various studies have suggested requirements for cross-linking of AChRs by antibody and for complement fixation¹⁵⁻²⁰. Such antibody effector functions are

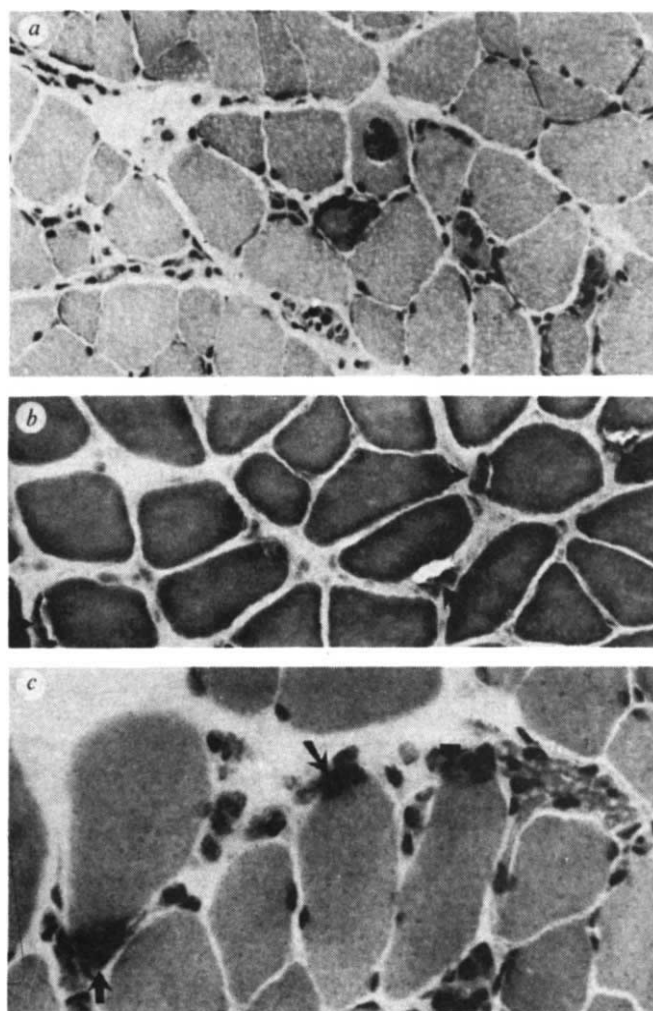


Fig. 2 Forelimb extensor muscle specimens from an animal injected 48 h earlier with antibody 56I. Fresh tissue was quick frozen, sectioned (9- μ m), and stained with haematoxylin and eosin (a), or for cholinesterase activity by either a modification of the Karnovsky method²² (b) or by α -naphthyl acetate esterase²³ (c). Some sections were also stained for acid phosphatase activity²⁴ (not shown). Mononuclear cell invasion, along with presence of necrotic fibres, is seen in a region shown, on serial section, to contain many end plates (a). Several cholinesterase-reactive regions (arrows) are separated from their muscle fibres (b). Direct observation identified these regions as cholinesterase-reactive by their distinctive brown gold colour. Invasion of cholinesterase-reactive regions (arrows) by mononuclear cells is seen along with disintegration of one region (arrow head) identified under direct observation by the distinctive dark red colour obtained with this technique (c). a, $\times 135$, b, c, $\times 90$.

Table 1 Effects of monoclonal anti-AChR antibodies

Hybridoma antibody (Class) [Specificity pattern]*	Disease score†	Amount injected (nmol)‡	Serum HA titre (log ₂)§	% Decrement at 5 Hz	m.e.p.p. amplitude (mV)
56I (IgG2b) [2]	3	4.8	16	20	0.49**
	3	3.6	11 (0.12)	26	0.50**
	2	1.8	ND	10	0.63
	0	1.0	10 (0.06)	incr.	0.51**
73G (IgG2b) [3]	3	3.6	14 (0.08)	13	0.58**
	3	3.6	13 (0.12)	incr.	0.64
	3	2.4	14 (0.09)	ND	0.53**
	0	0.5	10 (0.05)	3	0.56**
60D (IgG2a) [1]	0	1.8¶	17	incr.	0.71
	0	0.2¶	11	1	0.63
62J (IgG2a) [2]	0	2.2	16 (0.69)	incr.	0.65
	0	2.2	ND	incr.	0.64
57L (IgM) [2]	0	5.1	14 (0.03)	1	0.66
	0	5.1	6 (0.04)	incr.	0.77
77F (IgM) [4]	0	2.3	13 (0.12)	incr.	0.72
	0	2.3	4	incr.	0.81
P3-X63-Ag8 (IgG1)	0	0	0	incr.	0.80
	0	0	0	incr.	0.64

* Defined by effect of cholinergic ligands on HA titre⁸: 1, blockable by α -bungarotoxin; 2, partially blockable by cholinergic ligands; 3, increased titre in presence of nicotinic ligands; 4, unaffected by cholinergic ligands.

† 0, no clinical findings; 1, forelimb weakness; 2, weakness of all extremities and abnormal gait; 3, unable to support own weight.

‡ As determined by radioimmunoassay with ¹²⁵I- α -bungarotoxin-labelled AChR.

§ Values in parentheses are results, in nmol ml⁻¹, by radioimmunoassay.

|| incr. increment.

ND, not done.

¶ As determined by radioimmunoassay with ³H-AChR (ref. 11), since the binding of this antibody is blocked by α -bungarotoxin.

** $P < 0.01$ when compared with mean of 60D, 62J, 57L, 77F, P3-X63-Ag8.

primarily determined by heavy chain class or subclass. Both myasthenia-inducing antibodies, 56I and 73G, are of subclass IgG2b and should, therefore, be capable of diffusing into muscle, binding macrophages and fixing complement²¹.

The observations reported here establish that a single antibody species, in an otherwise normal organism, can lead to a severe immunologically mediated disorder. Loss of regulation of a single immunocyte clone may be sufficient, therefore, to cause an autoimmune syndrome.

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Physicochemical and immunological characterization of an HCG-like substance from human pituitary glands

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Human chorionic gonadotropin (HCG), or an HCG-like substance (HCG'), has been reported to be produced more widely in human than merely by placental tissue and by certain tumours^{1,2}. It has been difficult to detect low levels of HCG in the presence of human luteinizing hormone (LH) because the two are structurally similar and react similarly in most assays. Each is composed of two glycoprotein subunits (α and β), and the major difference between the hormones is an extra 35 amino acid residue at the carboxyl terminal of HCG β -subunit³. The availability of a radioimmunoassay using antibodies directed towards this sequence made possible the distinction of HCG' from LH in extracts of urine from normal postmenopausal women and in pooled human pituitaries^{4,5}. Although the existence of HCG' in either urinary extracts or pituitary fractions has been investigated extensively, on the basis of immunological, biological and physicochemical characteristics typical of HCG, the possibility that some proteases mimic the biological and immunological activities of HCG^{6,7} must be carefully considered. We describe here a series of isolation procedures, including a specific immunoaffinity chromatographic step and isoelectrofocusing, which not only separate HCG' from LH but also eliminate the effect of protease contamination. Furthermore, analyses of our purest HCG' have revealed that its isoelectric points, molecular size and immunological and biological activities are similar to those of HCG but distinct from those of LH.

Peptides corresponding to the carboxyl terminal sequence of HCG β -subunit are denoted here as s12, s30, s35 and t23 (refs 1, 8, 9). The numbers indicate the number of residues in the peptide (counting back from residue 145, the carboxyl terminal glutamine); s denotes synthetic and t denotes isolation from a tryptic digest of the β -subunit. A specific radioimmunoassay based on an antiserum to s35⁹ was used to measure HCG' in eluents of ion exchange chromatographs. Alternative antisera are specified in Figs 1 and 2 legends. Other assays used were an *in vitro* bioassay based on stimulation of testosterone production by mouse interstitial cells¹⁰ and a radioligand receptor assay using rat testis homogenates¹¹. The adsorbent for immunoaffinity column chromatography was prepared as follows. The γ -globulin fraction from a well characterized antiserum (H114)⁸ raised in rabbits against s30 was fractionated on a column of Sepharose 4B which had been covalently linked¹² to s12. After washing the column with 0.01 M Na, K-PO₄ and 0.15 M NaCl, pH 7.4 (PBS), the adsorbed antibodies were eluted with 0.1 M citrate buffer, pH 2.2. After dialysis and concentration by ultrafiltration, they were coupled¹² to Sepharose 4B. This immunoabsorbent preparation was able to bind 102 μ g of HCG (lot CR119) per ml of gel sediment.

The starting material for purification of HCG' was the freeze-dried follicle-stimulating hormone (FSH)-containing fraction

CM-1 which had been isolated by a standard procedure from pooled acetone-dried human pituitaries¹³. Specific radioimmunoassay revealed that HCG' was concentrated in this fraction, from which most of the LH had been removed. Further purification of FSH yielded an FSH-free side-fraction (designated DE-1) which was unadsorbed on DEAE-cellulose chromatography in 0.02 M potassium phosphate buffer at pH 7 (ref. 13) and which was eightfold more potent in HCG' than fraction CM-1. A small portion of fraction DE-1 (in the above phosphate buffer containing 0.2 M NaCl, 0.37 mM CaCl₂ and 0.37 mM MnCl₂) was fractionated on a column of concanavalin A-Sepharose. Less than 5% of immunoactive HCG' was unadsorbed. The bulk of the recoverable HCG' was apparently glycosylated as it could be eluted with 1 M α -methyl-D-glucopyranoside. Chromatography of DE-1 on Sephadex G-100 yielded a fraction (Seph-2B) in which HCG'-specific immunoactivity was increased a further twofold to a potency 1% as active as highly purified HCG (lot CR119). The peak elution volume of the immunoactivity suggested a molecular size larger than that of LH and similar to that of HCG. Fraction Seph-2B was obtained in a yield of about 1.6 mg per 1,000 pituitaries.

Immunoaffinity chromatography was used for further purification of HCG'. Fraction Seph-2B (1.5 mg) was applied to a column (0.9 $\times$ 10 cm) of Sepharose 4B linked to purified

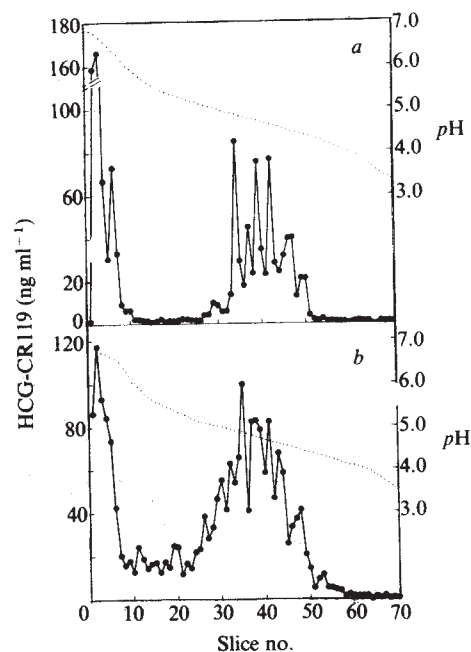


Fig. 1 Isoelectric focusing of fraction Aff-2. This was carried out with cylindrical gels in 6-mm i.d. Pyrex tubes¹⁷. Carrier ampholytes with pI ranges 2–4 (Accurate 42902) and 4–6 (LKB 1809–116) were each diluted to 4% (w/v) in 0.04 M L-histidine buffer, pH 7.74. Equal volumes were mixed to make a stock ampholyte solution. Polymerization mixture (2.35 ml per tube) contained 1% ampholytes, 5% acrylamide and 15% *N,N'*-diallyltartardiamide. Polymerization was started by addition of 0.015% (w/v) K₂S₂O₈, 5 $\times$ 10⁻⁴% (w/v) riboflavin and 0.05% (v/v) *N,N,N',N'*-tetramethylethylenediamine. The catholyte was 0.1 M L-histidine, pH 7.74, and the anolyte was 0.1 M formic acid, pH 2.29. HCG (CR119), 5.45 μ g, (a) and pituitary fraction Aff-2, whose immunoactivity approximated to 10 μ g of HCG-CR119 (b), were each dissolved in 100 μ l of 30% sucrose and applied to the gel. The electrofocusing was carried out at 200 V for 18 h. The pH gradient along the gels was measured by an automated procedure¹⁷. Gels were sectioned into 1-mm slices which were each extracted at 4 °C with 1 ml 0.05 M phosphate buffer, pH 7.4, containing 0.1% bovine serum albumin (BSA). Each extract was radioimmunoassayed using anti-HCG serum H80¹ (●—●), and major peaks were also bioassayed by measuring testosterone production *in vitro*.

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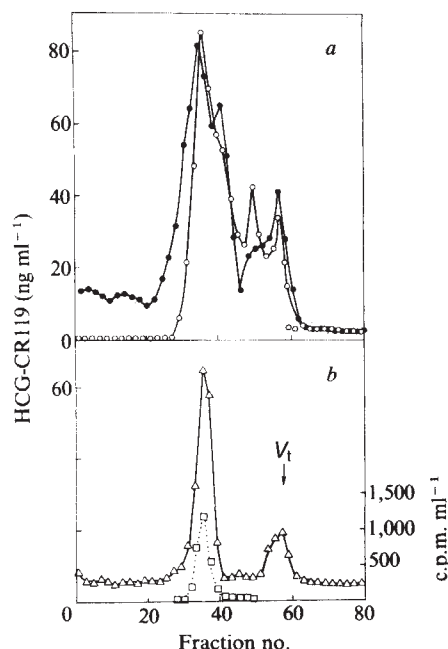


Fig. 2 Gel chromatography on Sephacryl S200 of material in the pooled major peak slices of Fig. 1b. A column of Sephacryl S200 (0.9 × 200 cm) was equilibrated with 0.05 M NH_4HCO_3 , pH 7.8, containing 0.1% BSA. Slices numbered 28–52 were pooled and extracted (as in Fig. 1 legend). The extract was desalted on a column of Sephadex G-25 (equilibrated with the above buffer) and freeze-dried. This material, plus a trace of ^{125}I -iodo-HCG, was dissolved in 2 ml of the buffer and applied to the Sephacryl column. The flow rate was 15 ml h^{-1} ; 2-ml fractions were collected. These were tested by radioimmunoassays using anti-HCG serum H80¹ (●—●) and anti-t23 serum H93¹ (○—○), as well as by a radioligand receptor assay (Δ — Δ) using rat testis homogenate¹¹. The elution of ^{125}I -iodo-HCG was monitored by its radioactivity ($\square$ — $\square$). The total column volume is indicated by V_t .

antibodies to s30 and equilibrated with the PBS buffer. The column was washed thoroughly with buffer and water, and adsorbed material (fraction Aff-2) was eluted with 1 M NH_4OH , neutralized with 1 M acetic acid and freeze-dried. This immunoaffinity procedure permits the separation of HCG' from LH and other substances by binding HCG' to the purified immunoabsorbent specific to the unique carboxyl terminal peptide of HCG β -subunit. Furthermore, this step should, we believe, eliminate possible pitfalls arising from contaminant proteases which have been reported to simulate HCG activities measuring displacement of ^{125}I -iodo-HCG, stimulation of steroid production or augmentation of adenylate cyclase activity^{6,7}.

To ascertain whether Aff-2 has physicochemical and immunological characteristics peculiar to HCG and biological activities typical of HCG, the following investigations were carried out. Fraction Aff-2 and purified HCG (batch CR119) were compared in isoelectrofocusing experiments (Fig. 1). Aff-2 contained components within the pI' range 4.4–5.1 (Fig. 1b), which is similar to the range for HCG as found by us (Fig. 1a) and by others¹⁴. This is further evidence that human pituitaries contain an HCG-like substance. When immunoreactive peaks were tested by the *in vitro* bioassay¹⁰, all were active in proportion to their immunoactivity. Using a single HCG (CR119) standard we obtained ratios of biological to immunological activities ranging from 0.3 to 0.6. The same range of ratios was obtained for HCG (CR119) in a parallel experiment. Reported pI' values for LH are between pH 7 and 9 (ref. 5). The nature of the higher pI' component which appears in both patterns is not known; it was not always detected in separate experiments carried out in the same conditions. If HCG' is a precursor of LH⁴ it is possible that the latter is formed during our experimental procedures and that it appears as the higher pI' component in

pattern b. Differences in sialic acid content of the various components, which are thought to be responsible for the electrophoretic heterogeneity of HCG¹⁴, may also account for that of HCG'.

To establish whether the acidic components of Aff-2 after electrofocusing had a molecular size similar to that of HCG, the acidic fractions (pI' 4.4–5.1; Fig. 1b) were pooled, desalted, freeze-dried, mixed with a trace amount of ^{125}I -iodo-HCG and chromatographed on Sephacryl S200. Chromatogram patterns were revealed by means of two radioimmunoassays and a radioligand receptor assay for HCG (Fig. 2). Each pattern contained a major peak, comprising 73–79% of total activity recovered, and the three peak maxima (corresponding to fractions 34 and 35) were each coincident with the maximum for radioiodinated HCG. Thus, most of the recovered HCG' with pI' 4.4–5.1 not only exhibited biological activity but also had a molecular size similar to that of intact HCG.

In conclusion, we have demonstrated that the substance purified from human pituitaries by Sephadex G-100 gel filtration followed by immunoaffinity chromatography using purified antibodies specific to the unique carboxyl terminal peptide of the HCG β -subunit, possesses pI' values within the same range as HCG and that the respective acidic components are biologically active in stimulating mouse interstitial cells to produce testosterone. The majority of these acidic components have the molecular size of HCG as revealed by gel chromatography. These data provide further evidence that an HCG-like substance is present in human pituitary extracts which differs from LH in its physicochemical and immunological characteristics. Furthermore, the HCG-like substance found in urinary extracts of postmenopausal women⁴ and a patient with Klinefelter's syndrome¹ could originate from the pituitary. Our data also suggest that pituitary HCG' is glycosylated and thus is different from the non-glycosylated HCG-like substance reported in very low concentrations in many normal tissues^{2,15}. However, there was no indication that the gonadotropic activity detected in the latter case was not due to proteases. Although it is possible that pituitaries of pregnant women or patients with neoplasm containing HCG or HCG' accidentally contaminate the pool of pituitary collection, the detection of HCG' in pituitary glands and in urinary concentrates^{4,5}, all from postmenopausal women thought not to secrete HCG, provides strong support that the normal pituitary gland contains HCG'. Larger quantities of highly purified pituitary HCG' will be required to determine whether it is identical to placental HCG or whether it is an immunologically related substance, possibly a precursor of LH⁴ (which is known to have some sequence differences in its β -subunit when compared with the same portion of HCG¹⁶).

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MATTERS ARISING

Does the C-terminal tetrapeptide of gastrin and CCK exist as an entity?

IT was reported by Rehfeld *et al.*¹ that the COOH-terminal tetrapeptide (Trp-Met-Asp-Phe-NH₂, abbreviated to G4 or CCK4), which is common to gastrin and cholecystokinin (CCK), exists in free form in pancreatic nerves, and may control islet hormone release. This report extends previous studies from the same group in which G4 was said to occur in pyloric antral mucosa in concentrations up to twice those of heptadecapeptide gastrin (G17), and in intestine and brain in concentrations considerably higher than those of the 33-residue form of CCK (CCK33) or its COOH-terminal octapeptide (CCK8). In these studies, particular importance was attached to the observation that after gel filtration of tissue extracts, a peak of material eluted in the position of G4 and reacted in radioimmunoassays using antisera specific for the COOH-terminus of gastrin and CCK. However, recent studies in our laboratory cast doubt on the interpretation of these data, and suggest instead that free G4 does not exist in significant amounts in pancreas or gut.

We have raised a rabbit antiserum to G4 conjugated through its NH₂-terminal amino group to thyroglobulin by glutaraldehyde. When used in radioimmunoassays with an ¹²⁵I-CCK8 label, this antiserum reacts almost equally with G4, G17 and CCK8, and can detect 10–20 pmol l⁻¹ of G4. This system offers a clear advantage for the estimation of G4 over the one used by Rehfeld *et al.*, because in the latter system G4 has a 30-fold lower immunochemical potency than CCK8 and CCK33. To estimate the true molar concentration of G4, it was, in effect, necessary for Rehfeld *et al.* to multiply the peak of activity eluting in the position of G4 by 30 compared with the other peaks. There is an obvious disadvantage in this method, for errors are also greatly magnified. Such a correction is unnecessary, of course, when G4, CCK8 and G17 and the other principal forms of CCK and gastrin have equal immunoreactivity. We have found that when hog pancreas is extracted according to the method of Rehfeld *et al.*, the total concentration of immunoreactivity measured with the G4 antiserum was 3.5 ± 0.8 pmol per g (mean, ± s.e., *n* = 4; G4 standard). Closely similar estimates (2.1 ± 0.4 pmol per g) were obtained with a second radioimmunoassay system using an antibody (L48, CCK8 standard) that, like Rehfeld's, reacts about 50 times less well with G4 than with CCK8. Fractionation on Sephadex G-50 indicated that over 80% of immunoreactivity in the extracts had

the properties of CCK8. These data indicate that G4 cannot occur in more than trace amounts in pancreas. However, when synthetic G4 was added to the tissue early in the extraction procedure in amounts comparable to those reported by Rehfeld *et al.*, the peptide was recovered in a yield of 73 ± 6%. In parallel studies we have also failed to confirm the presence of G4 in hog, rat or human antral mucosal extracts, which according to Rehfeld *et al.* contain G4 at about 15 nmol per g; instead, we have consistently found a major peak of immunoreactivity corresponding to G17, confirming numerous previous studies.

We conclude from our work that free G4 does not occur in significant amounts in pancreas or gut. The high concentrations of G4 reported by Rehfeld *et al.* reflect the fact that a minor component of gastrin-CCK immunoreactivity is interpreted as G4 and estimates of its molar concentration corrected to allow for the low potency of G4 in the assay system. This correction might conceivably be justified if G4 was proven to exist naturally by isolation and full chemical characterization; this has not been the case. Until the material described by Rehfeld *et al.* in pancreas and elsewhere is isolated and characterized, its true concentration in these tissues cannot be determined. The issues raised here serve to emphasize the very great caution that needs to be exercised in identifying and determining substances by gel filtration and radioimmunoassay, when the material in question has not been isolated.

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1. Rehfeld, J. F. *et al.* *Nature* **284**, 33–38 (1980).

REHFELD ET AL. REPLY—First, we note that the total CCK immunoreactivity in pancreatic extracts measured by the G4 antiserum, 3.5 pmol per g, is of the same order of magnitude as that reported by us¹ (6.8 pmol equiv. CCK8 per g). It is interesting also that the L48 antiserum, which reacts less well with G4, measures lower concentrations (2.1 pmol per g) than the G4 antiserum, suggesting that G/CCK4 is indeed present in the pancreas.

Second, Dockray has previously reported² that the mammalian brain contains significant amounts of a third immunoreactive component (BP III) with a size corresponding to G/CCK4 or G/CCK6. He measured this BP III peak

by four different C-terminal specific antisera (including our ab. 2716). Using CCK8 as standard, the BP III peak constituted 12% of the CCK8-like peak in hog, 31% in dog and 18% in rat cerebral cortex (Table 2 in ref. 2). The BP III peak should be easily measurable by the new G4 antiserum. We are surprised that Dockray has not discussed these earlier results in the light of his new assay findings.

Third, we can only speculate on the reason for the discrepancy between our data (supported by Dockray's earlier data²) and those of Dockray and Gregory. (1) Microheterogeneity of the natural tetrapeptide (in analogy with the microheterogeneity of CCK octapeptide³) might explain^{4,5} the failure of the new assay to measure G/CCK4-like material. (2) Several samples of synthetic G4 received by us from other laboratories have been found to be grossly impure, and even the purest contain minor impurities—might the new G4 antiserum of Dockray and Gregory have specificity for these impurities? (3) In the raising of antibodies, we question the use of G4 conjugates prepared by means of glutaraldehyde. The presence in G4 of N-terminal Trp complicates the normal reaction with aldehydes, and we would anticipate that conjugation would be through a carboline rather than the terminal amino group. If this is so, the specificity of antibodies raised to such conjugates might be abnormal.

In conclusion, we consider the evidence to be in favour of the presence of a tetrapeptide in brain, gut and pancreas, but final confirmation must come from isolation and sequence work.

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BOOK REVIEWS

Most orthodox heresy: Jensen on IQ myths

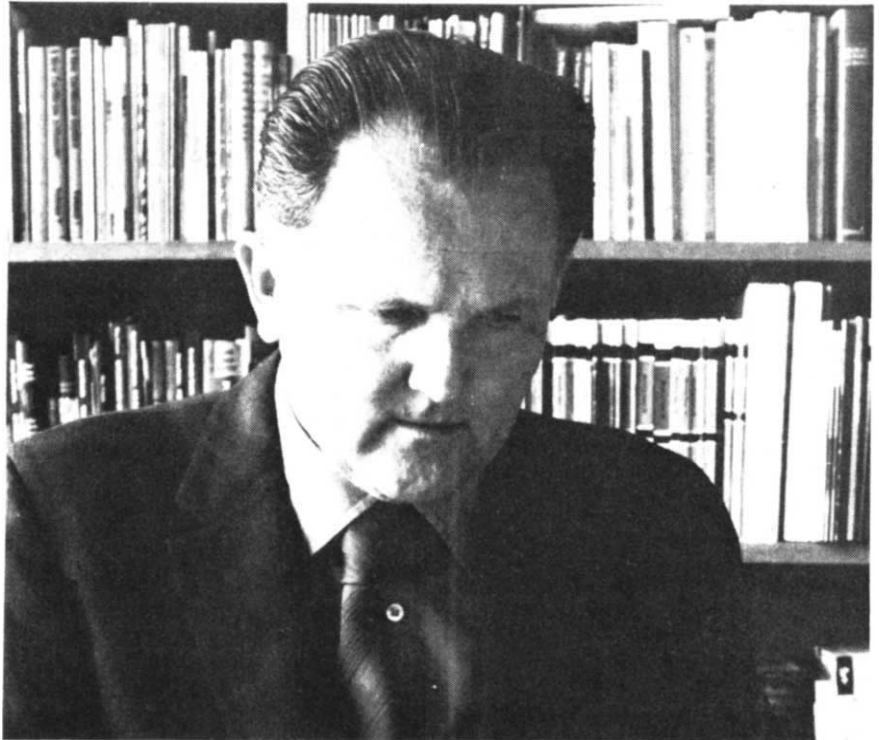
Steve Blinkhorn

Bias in Mental Testing. By Arthur R. Jensen. Pp.800. (Methuen: London/Free Press: New York, 1980.) £15, \$29.95.

CONSPIRACY theory ranks high in popularity among theories of psychological testing. According to this view, psychometricians, whether wittingly or not, have succeeded in constructing devices which systematically and unwarrantably discriminate against non-whites, lower social classes and possibly females. There is a popular belief that intelligence (mental ability, scholastic aptitude, mental maturity and so on) tests are constructed using scholastic achievement in a white middle-class educational system as a criterion and that devious means are adopted to force scores to a normal distribution. Accordingly, such tests are inappropriate for non-whites, for working, rather than middle-class, individuals and so on. The appropriate action is therefore either not to use them at all or to correct the inherent bias by using lower cut-off scores for, or adding a constant to the scores of, the disadvantaged groups.

Added impetus to the propagation of this point of view has come in the aftermath of the Burt affair, and phrases such as "the IQ myth" and "the new assault on equality" have gained currency. One of the few topics on which most psychologists (and not only psychologists) seem to feel entitled to pronounce is the validity or otherwise of so-called IQ tests. Sadly, such pronouncements do not always benefit from the technical competence and sense of history which make for an informed judgement. There is a notion abroad that what tests measure and how fairly they discriminate can be determined by inspection, the implication being that psychometricians are peculiarly insensitive to what is patently obvious or deliberately, and for the worst of motives, disingenuous in the pursuit of their craft.

The whole question of inherent bias in mental tests has been brought to a new crisis point by a series of court judgements, which have handed down verdicts as to whether or not particular tests are biased. The notorious 15 IQ points difference between mean scores of blacks and whites in the USA gives the topic social, moral and political importance. If this difference is the result of test bias, then something ought



Arthur Jensen, "scholarly and dispassionate".

to be done about the tests. If, on the other hand, the tests merely reflect a true difference, however caused, then no amount of advanced psychometrics will save an unhappy state of affairs.

Jensen's description of how, as a student, he "came to believe that nearly all standard IQ tests were grossly biased against virtually everyone but the white middle class" is an unexpected entrée to a book which amasses an overwhelming weight of evidence to the contrary. He then proceeds to spend nearly half the book developing the statistical and methodological basis for an examination of test bias. There is implicit in his treatment of classical test theory a criticism of the standard of public debate on test bias. Too many psychologists are either ignorant of, or dismiss as statistical conjuring tricks, elementary procedures such as correcting correlation coefficients for restriction of range or for attenuation by unreliability, or regard predictive validity coefficient of the usual magnitude (0.3 to 0.5) as hopelessly small and indicative of little utility. On the assumption that all interested parties were thoroughly acquainted with basic psycho-

metrics, the book could have been half its actual length.

On the question of bias itself, Jensen is remarkably up to date with the current state of theory and practice. The distinction between bias (a statistical concept and a matter of fact) and unfairness (which involves a value judgement) is well made, and the fact that different definitions of fairness conflict when made operational in terms of the practical use of tests is driven home. The kernel of Jensen's thesis is to be found in Chapters 10 and 11, in which the empirical evidence of test bias (1) in predicting occupational and educational success and (2) in the internal structure of tests, is reviewed. Much of this material is counter-intuitive. It is not the case that for a given test score blacks perform better at work or at school. On the contrary, using prediction equations based on white groups may disadvantage the whites, in that blacks have to do as well or better on the tests to do as well on the task. Nor does the notion that verbal tests yield lower scores for blacks than do non-verbal tests, stand up to scrutiny. Finally, there is no evidence from the internal structure of tests

that they are measuring something different in the different racial and social groups.

In short, Jensen provides methods for detecting bias, collates a large amount of empirical evidence and justly concludes that there is no substantial, replicable evidence of inherent bias in commonly used tests where native-born English speakers are concerned. He also disposes of several widespread myths about tests, such as the so-called Pygmalion (teacher expectancy) effect. There is a willingness evident in the psychological community to accept criticisms of psychometric techniques on blatantly *ad hoc* grounds, and a supreme unwillingness to pay attention to the large body of empirical evidence which supports the utility of tests, in particular in cutting across social and ethnic groupings and revealing talent where it is least expected (which, after all, was the original reason for their introduction).

Jensen has neither time nor patience for the overuse and misuse of tests, however. His last chapter is an indictment of malpractice which ought to be required reading for all those who actually enjoy using tests or like to treat IQ as the criterion of personal worth *par excellence*.

Undoubtedly many will feel that by restricting himself to a rather technical brief Jensen is missing the point. The abstractness for which test constructors often strive, they may point out, is itself a cultural variable, highly prized and cultivated in the white middle-class. The format of tests reinforces certain values (accuracy at speed, seeking to generalize, solution of problems in the abstract rather than the concrete) which may be dominant in white middle-class culture.

There follow from this two questions. Is it the case that the kinds of abstract reasoning abilities implicated in, say, Spearman's view of intelligence can be consistently assessed? Is it the case that an individual's capacity to deal effectively with his world depends on such skills? If the answer to the first is affirmative, as Jensen claims and by and large demonstrates, the answer to the second is by no means an obvious yes. Wechsler has defined intelligence as "the capacity of an individual to understand the world about him and his resourcefulness to cope with its challenges". Such a view clearly involves the nature of the world in which an individual exists, and non-intellective characteristics of the individual as important elements in the determination of intelligence.

Now, tests constructed in line with the views of both Spearman and Wechsler intercorrelate highly. This is hardly surprising, given the importance placed in Western society on intellectual skills, for we live in a society where some social security forms demand a higher level of reading skill than the *Times*. But the distinction needs to be maintained, not least because whilst a given level of

intelligence in Spearman's terms may imply capacity to cope with the business of living, the reverse need not be true. One wonders whether the high value placed on intelligence in our society is a universal feature, not so much in remote tribes as in ancient Egypt and Rome, or to choose a more immediate instance, in China during the cultural revolution. That is to say, could we choose to organize our society so that differences in intelligence are unimportant, and if so would that kind of society be successful, stable and desirable?

If, as Jensen shows, the accusation of test bias is unfounded, then the truth of the matter is that differences in achievement reflect the extent to which society demands the abilities assessed by the tests. Whether these demands are proper, necessary and fair is entirely open to question, but fiddling test scores to make racial differences vanish is as unworthy as the exaltation of IQ as the sole criterion of personal worth. It neither promotes the interests of those in sympathy with whom it is done nor protects them against the failure it makes more likely. It makes as much sense as subtracting 8 IQ points from the scores of myopics, on the grounds that this is the mean difference between myopics and non-myopics, and clearly myopics are advantaged in that their condition makes them read more books and play less football.

There is a widespread notion that since tests necessarily involve specific tasks, IQ is merely a matter of specific learned skills of a particular kind. Yet such evidence as there is suggests that coaching in specific skills has little transfer value. The point of intelligence testing is not whether an individual can solve problems having a certain logical structure, but whether he can identify the logical structure and find strategies to solve them. Many tasks can be made easier by making their structure more patent and immediately concrete: much of the recent work critical of Piaget has adopted this approach. Such studies point to ways of bringing a wider range of performances within the capacity of the individual, and have immediate relevance to education and training. Their relevance to the study of intelligence, seen as a high-level ability to detect the structure of problems and organize lower-level strategies towards a solution, is however seriously in doubt.

This book is informative, refreshing and a valuable contribution to the literature; its worth as a compendium can hardly be overrated, and it may well find a role as a textbook of psychometrics for the clarity of its exposition of classical test theory. Its scholarly and dispassionate tone will come as a surprise to those who know of Jensen only as a leading figure in anti-test demonology, however out of sympathy they may be with its content.

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Understanding nuclear astrophysics

Bernard Pagel

An Introduction to Nuclear Astrophysics. By Jean Audouze and Sylvie Vauclair. Pp.168. (Reidel: 1980.) Hardback Dfl.75, \$39.50; paperback Dfl.40, \$19.95.

SINCE the 1920s, the development of astrophysics has been closely associated with advances in nuclear physics which have led to our current understanding of stellar energy sources, stellar evolution, nucleosynthesis and nuclear cosmochronology, and to continuing attempts to understand the distribution of the chemical elements in the light of ideas about cosmology and the evolution of galaxies.

These topics are engagingly presented by two well-known French astronomers in this book, which is written at a level suited to first-year undergraduates, but will also be appreciated by graduate students and others desiring an up-to-date introduction to the subject. Succinct descriptions of the basic interactions of physics and of the observable Universe are followed by outlines of big-bang cosmology, stellar evolution and the abundances of the elements in the Solar System, stars and cosmic rays. The remainder of the book gives somewhat more detailed accounts of specific theoretical topics: nuclear reactions and the various nucleosynthesis processes currently considered significant, Solar System and galactic nuclear time-scales and the chemical evolution of galaxies. A brief concluding chapter sketches historical developments and a few outstanding problems.

By far the strongest feature of this book is the nuclear physics, which is carefully and clearly described at the appropriate level. Other aspects are more sketchily treated. This does not matter too much for the observational part, which is adequate as a background to the theory apart from occasional minor infelicities such as a reference to quasars as having "the same optical properties as the stars". A more serious gap is the lack of a coherent account of the non-nuclear aspects of stellar structure and evolution, which makes it difficult for the reader to envisage the context in which the nuclear reactions operate. The chapter on nuclear chronologies and the origin of the Solar System is especially good, but the one on chemical evolution of galaxies shows signs of over-hasty preparation and proof-reading — some equations (including one credited to myself!) are either nonsensical as written or inconsistent with the accompanying text; inconsistent and undefined symbols also occur. (There are also a few minor errors of fact here: the abundance ratio N/O is not as high in the Magellanic Clouds as in the solar neighbourhood and the simple model of

galactic chemical evolution does not predict that we should see stars with *literally* zero metal abundance.) The English is generally adequate, though noticeably less than perfect. Finally, prospective purchasers should be warned that I have come across a copy (not my review copy) that is misbound, with several pages in the wrong order.

In short, a curate's egg of a book in which the largely excellent intentions of the

authors have been frustrated by insufficient attention to detail at different stages of production. The broad sweep of the subject is well presented, though, the illustrations are good and a useful set of references is given, so that on balance this is a welcome and useful book. □

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Number 3 in the Enzymes dynasty

Athel Cornish-Bowden

Enzymes. Third edition. By Malcolm Dixon and Edwin C. Webb. Pp. 1116. (Longman: London/Academic: New York, 1979.) £25, \$49.50.

WHEN THE first edition of this book was reviewed in *Nature* (182, 969-970; 1958) it was described as "one of the finest books that has appeared on the topic of enzymes". Two editions and 22 years later this is still true; there have been other excellent books on enzymes, but none with the breadth of this one. Despite the long period since the second edition appeared in 1963, the new edition resembles its predecessors surprisingly closely and has essentially the same organization of material. The authors have included an astonishing amount of useful information in what is still a single volume. This is especially remarkable in view of the amount of space they have devoted, mistakenly in my view, to the Enzyme Commission's classification system for enzymes. More than 300 pages are given over to a description of its principles and a virtually complete list of known enzymes. Surely a better use could have been found for this space.

Another section of questionable

usefulness, one that must surely have added substantially to the price of the book, is the "Atlas" of crystalline enzymes, which consists of 25 pages of reproductions of photomicrographs. As this is now relegated to close to the back of the book it would seem that the authors recognize that readers no longer gasp in wonderment at the knowledge that enzymes can be crystallized.

In other respects the authors have displayed an admirable sense of what is worth including and what is not. The early chapters are concerned with practical matters and include much useful advice without becoming enmired in experimental detail of no general interest. The discussion of steady-state enzyme kinetics is especially good and almost justifies purchase of the book by itself. Rather oddly, it is not all in one place but is divided between Chapters 4 and 8, which account for about a quarter of the book. Obviously there are some points of detail where one might quarrel with the treatment, but in general it is balanced and thorough. These two chapters also contain an account of transient-state kinetics, but one senses that this is not a major interest of the authors. The other chapters, concerned with specificity, mechanisms, cofactors, protein structure, protein biosynthesis and enzyme biology, are also of a high standard. Of these, protein biosynthesis is perhaps one topic that might have been omitted, not because of any lack of importance, but because it has

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developed into a subject rather separate from general enzymology and I doubt whether its devotees will look for an account of it in a book about enzymes.

Finally, a word about production. I was pleased to be sent a copy of the American edition for review, as this is soberly and robustly bound. By contrast, the British publishers should be ashamed of their covers, which are not only ugly but are also far too flimsy for a book of 1100 pages.

Athel Cornish-Bowden is a Lecturer in Biochemistry at the University of Birmingham.

Scientific terms by numbers

Alan Isaacs

Longman Dictionary of Scientific Usage. By A. Godman and E.M.F. Payne. Pp.684. (Longman: 1980.) Flexi £4.35, \$14.95.

FOR THE casual user of a scientific dictionary — someone who needs to look up the meaning or spelling of a word from time to time — this is not the book to buy. To find the meaning of a word in this dictionary you have first to read the

instructions, then you have to look in the index for a two-letter set code that will identify the field of science of the word and for a three-figure number to find the position of the word in the set. Memorizing both the set code and the number, you then search the body of the book for the appropriate set. The alleged advantage of this cumbersome system is that the book is designed specifically for the non-native speaker of English who will benefit from the thesaurus-like arrangement in which terms that are "closely related in meaning or subject area" are grouped together. Even if foreign learners have more patience and perseverance than native speakers, this system has its dangers. If you look up *free electron* in this dictionary you will be

directed from the index to the section on radio terms (code NM). There you will be told that it is "an electron in a crystal which [*sic*] is free to move under the influence of an electric field". The user, if he relies on this dictionary, will never know that free electrons also exist in gases. This example highlights the hazards of this kind of coding.

In general, though, the definitions appear to be clear and accurate, and there are plenty of useful collocations and cross-references to help the foreign learner (once he has mastered the meaning of the cross-referencing arrows pointing East, North and South). It is such a shame that most compilers of dictionaries for non-native speakers feel obliged to clutter their books

with redundant information (the new *Chambers Universal Learners' Dictionary* is a notable exception). Will a foreign scientist or student really be helped by seeing An or H against a word? Even if he looks in the front matter, where he will learn that the former denotes a non-

gradable antonym and the latter a hyponym, will he be any the wiser? If I were a foreign scientist I would want a dictionary that tells me what I want to know simply, by having the information available in its alphabetical place. I am not sure that I would want to be confused with

coded answers to questions that I am never likely to ask. Hyponym indeed! □

Alan Isaacs, a former teacher of scientific English to foreign students, is editor of the Penguin Dictionary of Science and Longman's New Dictionary of Physics, and science editor of Collins English Dictionary.

RNP particles and the virus club

B.S. Cox

Viruses and Plasmids in Fungi. Edited by Paul A. Lemke. Pp.680. (Dekker: 1979.) SFr.110, \$55.

MANY fungi harbour ribonucleoprotein particles consisting of a spherical or polyhedral capsid-like structure 35 ± 10 nm in diameter, containing one or more molecules of double-stranded RNA. The particles are rather reminiscent of reovirus.

They are a remarkable group of objects. In the first place, it appears to be quite impossible to infect any fungus with them. The lengths to which numbers of mycoviologists have gone to demonstrate this rather fundamental property of a virus have been extraordinary, both for the man-hours spent and the total lack of success. Secondly, without exception, they are symptomless. Of course it is rather difficult to connect the symptoms of a disease with a virus if one cannot infect the host with it;

and the energetically-researched "La France disease" of cultivated mushrooms may indeed be caused by the two or more 'viruses' associated with it. On the other hand, of course, the disease may cause the viruses. 'Virus' is often found, apparently, in healthy mushrooms.

Not all of the dsRNAs of fungi are without phenotypic effects. Cytoplasmically inherited dsRNAs produce the 'killer' toxins of many species of yeast and of the smut fungus, *Ustilago maydis*. They also apparently cause the fascinating non-pathogenic variant of *Endothia parasitica*. This blight has deprived two generations of Americans of their native chestnuts for the Thanksgiving stuffing. The hypovirulence of the variant is invasive and suppressive, and can be used not only to limit the spread of the cankers caused by virulent strains on *Castanea* but actually to depress the virulence of the pathogen itself.

Cryptic or not, these RNP particles of fungi are curiosities. They are extremely widespread, occurring in all major groups of fungus including water moulds. They form a strikingly homogeneous group of

organelles. Homogeneity, universality and non-infectionness suggest a very ancient common origin, but how have they been maintained? Is it possible for a non-essential, wholly cryptic self-replicating particle to survive in such a specialized environment without the means of spreading by infection? The killer (M) dsRNA of yeast depends for its maintenance on 30 nuclear genes: one mutation and it's gone. How does it survive? The L-dsRNA has not been found to depend on any nuclear genes. Why not? Do the dsRNAs do something useful, or is the killer/immunity property a self-selecting mechanism? If so, do all fungi possess it?

Although neither answers to these questions nor, indeed, the questions, are to be found in this book, there are two clear and succinct reviews of the genetics and molecular biology of the RNP particles of yeast, *Ustilago* and *Endothia*. There are also excellent accounts, sometimes commendably brief, of an assortment of non-RNP particles, DNA plasmids, bacterial or rickettsia-like structures and even a couple of cytoplasmically-inherited conditions with no particle to call their own (senescence in *Podospira* and meiotic failure in *Coprinus*). For the rest, there are detailed, rather self-indulgent articles cataloguing biophysical and ultrastructural studies of RNPs, speculations about their life-histories, a methods paper without protocols, many more or less indistinguishable electron micrographs and many a discussion about what these RNPs should be called. The general opinion is that they should be awarded the accolade 'virus', perhaps by broadening the category of admissible members. I can't help feeling that this obsession with joining the club has rather diverted attention and effort away from the problem of what they really are. Much of the repetitive material about functionless particles could have been condensed into a single review article (Chapter II covers all the ground) and perhaps some space given to the many other cytoplasmically-inherited determinants of fungi which have phenotypes but no physical basis yet identified. Visibility in the electron microscope is not the only passport to respectability.

The book is a little out of date but is, nevertheless, for some topics, a very useful source. □

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Jewish genetics

Kurt Hirschhorn

Genetic Disorders among the Jewish People. By R.M. Goodman. Pp.493. (Johns Hopkins University Press: 1979.) \$32.50, £17.50.

THE genetics of the Jewish people have received a great deal of attention over the past few years. This is primarily due to the virtually unequalled opportunity created by the population dynamics of the state of Israel. Previously isolated groups of Jews have been immigrating to Israel, where they continue a certain degree of inbreeding and are beginning to outbreed with other Jewish groups. Driven by the tradition of Israeli research on human genetics, particularly under the leadership of the late Drs E. Goldschmidt and C. Sheba, a new and large generation of human geneticists has been quick to take advantage of this temporary opportunity.

Three recent publications summarizing these data as well as studies done outside Israel have been *The Genetics of the Jews* by A.E. Mourant, A.C. Kopec and K. Domaniewska-Sobczak (Clarendon:

1978), *Genetic Diseases Among Ashkenazi Jews* edited by R.M. Goodman and A.G. Motulsky (Raven: 1979) and the book reviewed here. The first of these is primarily a tabular accumulation of gene frequencies in various Jewish groups and contains much useful data, while the second represents the proceedings of a symposium with many excellent reviews.

This present book is a *tour de force* which pulls together a mass of reports and develops a comprehensive description of the status of these studies. One could argue with some details such as interpretations of ancient history and the inclusion of a number of genetic diseases found in Jews with "Jewish genetic diseases", and there are occasional lapses in clarity. None of these criticisms should detract from the fact that this is a major and useful book for all human geneticists as well as students of Judaism. This is a field which is applicable to genetic centres wherever Jewish patients may appear and which is certain to grow over the next 20 years. The book also provides a general model for genetic studies in populations, and is therefore recommended for a wide audience. □

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